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STUDIES ON THE ACTIVITIES OF THE INFECTIVE LARVAE OF NECATOR AMERICANUS.

DISSERTATION

Submitted to the Director of the School of Hygiene and Public Health of the Johns Hopkins University in Conformity with the requirements for the Degree of Doctor of Science in Hygiene.

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June 1923.

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General Introduction

Our knowledge of the activities of mature, infective larvae of the hookworm has been founded almost entirely upon laboratory experiments and observations. Length of life studies on the larvae were made by keeping sheathed forms in water, usually in closed containers. These experiments, for the most part, were carried on in the sub-tropics or in temperate regions. Under these conditions, they have been reported to have lived for more than one year, and it was believed that they could live as long, or even longer in the soil. The conception of the danger of the spread of the larvae in the soil was founded largely upon studies of the behavior of ensheathed larvae when mounted in water, or water and soil, on glass slides, and examined microscopically. Their lively movements under these conditions established the idea that their habits were similar in the soil and that large areas could become infested from small centers.

Due to many difficulties encountered, definite information on the activities of hookworm larvae in the soil was almost unattainable. However, after the discovery by Baermann in Sumatra of an apparatus with which hookworm larvae could be isolated from considerable quantities of soil, the entire attack on the study of the hookworm has been changed. This apparatus,

with slight modifications, has made it possible to accurately study the development and activities of infective hookworm larvae in their natural environments. Such studies were carried on from March 1921 to April 1923; under the tropical conditions of Trinidad and Porto Rico, West Indies, during the summer of 1921 and 1922, and in Baltimore during the falls and winters of this period.

These studies have shown that many of our former conceptions concerning the biology of mature hookworm larvae are erroneous and that a revision of these ideas is necessary. The length of life of mature larvae was found to be much more limited than was formerly supposed and under tropical conditions, an almost complete dying out occurred in less than two months. It was also found that mature larvae become unsheathed in the soil, a phenomenon which was supposed to occur only at the time of penetration of the skin of the host. This casting of the sheath was found to be significant in that it immediately renders the larva more susceptible to adverse conditions and functions in reducing soil infestation. It was also shown that the infective larvae do not migrate from places of development.

The difference between these results and those of former investigators can be explained by the fact that the use of the Baermann isolation apparatus made it possible to study

the larvae under natural conditions.

I wish here to express my thanks to the various members of the Trinidad and Porto Rico expeditions for their cooperation and suggestions during the course of this work; and to the International Health Board of the Rockefeller Foundation for making the studies in the tropics possible.

To Dr. William W. Cort, under whose direction this work has been carried on, I wish to express my appreciation for his continued interest and helpful criticism.

I. EXPERIMENTS ON THE MIGRATION OF HOOKWORM LARVAE IN SOILS.

Historical Account.

Until Baermann (1917) devised an apparatus with which hookworm larvae could be successfully isolated from the soil, little or nothing was known of their activities under natural conditions. Throughout the literature, one is impressed with the idea that the infective hookworm larvae may actively migrate for considerable distances, and may thus give rise to infestation far from the place where the eggs were originally deposited.

Claude A. Smith (1903, p. 710) recorded that the larvae dart through a sandy soil at a lively rate for a few minutes to half an hour or more, and then straighten out and lie perfectly still for a corresponding length of time. In the report of the Porto Rico Anemia Commission (1904, p. 121) the statement was made that rain causes a distribution of larvae, and that this, together with their natural motility, may spread nests of soil infestation over a considerable area. Nicoll (1917, p. 163) in his discussion of the hookworm larvae, under natural conditions, made the following statement:

"The larvae now measure about 0.6 mm. in length and are very actively motile. Unless they have been washed away previously from the faeces, they now proceed to migrate to a more suitable locality, namely: the nearest water pool or patch of moist ground. Provided even a trace of moisture be present, they are capable of traversing considerable distances and may thus give rise to infection far from the place, where the faeces were originally deposited. How far and how rapidly they may wander in this fashion is not definitely known, but judging from laboratory experiences their rate is probably not less than five feet per hour, so that in the course of 24 hours, they may have wandered 40 yards. This means that an area of 5,000 square yards might become infected within 24 hours after the larvae started to migrate, i.e., within a week after the faeces were deposited."

Chandler (1918, p. 259) pointed out that continual traveling in a straight line would probably never occur, but that a single stool containing hookworm eggs could easily infest at least several square yards. Similar statements on the spread of hookworm larvae were also made by Ozzard (1909, p. 779).

The first intimation that the infective hookworm larvae might be limited in their distribution throughout the soil was made by Price (1910, p. 205). He stated that in his

opinion sub-soil pollution was not an important factor in hookworm disease, because it appears inconceivable that hookworm larvae will pass for any considerable distance through a sand and gravel sub-soil.

Based on the work of these authors, the opinion has become current that the infective hookworm larvae move out from centers of infestation and spread themselves over wide areas. This conception is founded largely upon the study of the behavior of the encapsuled larvae when mounted in water, or water and soil, on glass slides or in watch glasses, and examined microscopically. To what extent the migrations of the larvae occur from definite infested centers in soils of various types and under varying conditions, has not yet been demonstrated. The experiments which will be described in this paper were carried out in Trinidad, British West Indies, from May to September, 1921, in order to gain information on this very subject.

Material and Methods.

The larvae used in these experiments were obtained from cultures 5 to 8 days old. A detailed account of the method used in the preparation of the cultures is given in number two

of this series (Cort, et al., 1922, p. 6). The larvae were carefully counted under the microscope, the 16 mm. objective being used, when the hookworm larvae were mixed with other nematodes, and a lower power, when they were in pure culture. As Ancylostoma duodenale is very rare in Trinidad it may be assumed that the larvae obtained were those of Necator americanus.

Two sizes of pans were used in the experiments on migration, vix.: (1) round tin dripping pans, 13 inches in diameter and 4 inches high, and (2) pudding pans 6 inches in diamter and 2 inches high.

The soils in which these larvae were studied were selected from the district in which these experiments were carried on, the Naparima District, which is in the south central part of Trinidad. They were chosen from areas free from soil pollution, and tested in the laboratory with the isolating apparatus for the presence of hookworm larvae and free-living nematodes. None of the samples were found to contain hookworm larvae and where free-living nematodes were numerous, the soil was heated to 150° F. This temperature was found to be sufficient to kill all living organisms present without altering the composistion of the soil itself.

The migrations of the hookworm larvae were studied in five distinct types of soils. Type 1 was a black, rather heavy, clay loam which contained so small stones. Type 2 was identical with type 1, except that it contained a greater amount of humus and was lighter. These two soils are classified by Williams (1921, pp. 106-109) under the Maparina Marls or the Black Soils of the Maparimas. Type 3 was also a heavy clay loam distinguished by its red color. It differed from types 1 and 2, chiefly in its chemical rather than mechanical composition being deficient in lime and having a high percentage of iron. Type 4 was a fine sand and clay mixture, light when dry or slightly moist, but heavy and compact when wet. Type 5 was a loose reddish sand, containing only a small amount of clay.

Experiments on Active Migration.

Table 1 gives the data from a series of twenty experiments to test the active migration of infective hookworm larvae under a variety of conditions. In all the experiments, summarized in this table, the soils were placed to the depth of 1 cm. in the 13-inch dripping pans. The center was then determined, marked by a cord circle 1 inch in diamter, and the larvae placed with-this circle. This area was termed Zone 1, or the center of infestation. When I wished to close an experiment the soil was further divided into Zones 2, 3 and 4. This was accomplished with an apparatus

similar to a doughnut or cooky cutter. It consisted of three stationary tin bands of sufficient circumference to make each of these zones two inches wide. The soils of the three outer zones were transferred separately to units of the large isolation apparatus, without withdrawing the "cutter." The danger of mixing the soil of one zone with the next was thereby reduced to a minimum. Two units of the isolation apparatus were used for zone 4, to keep the amount of soil in each unit approximately equal. The cutter was then removed, and the soil from the infestation center transferred to a unit of the small isolation apparatus.

In describing the moisture content of the soils the term "moist" indicates a soil which had a slightly sensible wetness, while the term "saturated" denotes a soil which had been thoroughly soaked and its surface fairly covered with water.

It will be seen from an examination of Table 1, that the larvae did not manifest the migratory habit which has been attributed to them, since in no instance were they found as far as four inches from the center in which they were placed. In only 4 experiments (Table 1, nos. 5,7, 17 and 19) were larvae recovered from the third zone, and in each of these cases less

than 2 per cent. of the total number of larvae recovered had reached this zone. It is also apparent that neither the type of soil nor the presence of vegetation enter as factors in the extent of active migration of hookworm larvae. A thick grassy sod was not available at the time of these exp riments, and in order to study the larvae under this environment the soil - clay loam - was first heavily sown with a mixture of oats and wheat. The larvae were not added to the infestation centers until the grass had formed a heavy root system, and had reached a height of 3 to 4 inches. No perceptible difference was observed in the extent of migration of the hookworm larvae, when exposed to direct sunlight under temperatures varying from 93° F. to 101° F. (Experiment 20, Table 1). The migration pan was set out in direct sunlight during the day and returned to the laboratory at night. At no time was the soil allowed to become dry. As soon as a dry crust appeared on the surface it was evenly sprinkled with water, thus keeping it as uniformly "moist" as possible. While the sunlight had no apparent influence on the extent of spread of the larvae away from the infestation center, it had an indirect effect on the activities of the larvae within that area which is discussed in number eight of this series.

TABLE 1.

Experiments on the active migration of hookworm larvae. All these tests were made in the thirteen-inch pans.

Experiment Number	No. larvae in center of infestation	Type of soil	Conditions	Time larvae remained in soil	No. of H.W. larvae recovered from Zone 1	Zone 2	Zone 3	Zone 4
1	800	Clay loam	Moist, shade	15 hours	409	15	0	0
2	1,000	Clay "	" "	16 "	583	274	0	0
3	1,800	Loose loam	" "	40 "	1,395	128	0	0
4	1,000	Loose "	" "	15 "	460	406	0	0
5	1,000	Clay loam	" "	7 days	236	84	1	0
6	1,000	Clay "	" "	7 "	478	39	0	0
7	512	Clay "	" "	11 "	89	145	3	0
8	1,100	Clay "	" "	21 "	13	5	0	0
9	650	Clay "	" "	42 "	1	0	0	0
10	525	Red clay loam	" "	15 hours	363	69	0	0
11	500	" " "	" "	22 days	6	3	0	0
12	277	Clay loam sod	" "	7 "	60	10	0	0
13	392	" " "	" "	21 "	2	3	0	0
14	361	" " "	" "	30 "	1	1	0	0
15	426	" " "	" "	37 "	1	0	0	0
16	498	Sand	" "	10 "	51	40	0	0
17	451	Sand	" "	22 "	77	16	1	0
18	498	Sand	" "	29 "	51	40	0	0
19	1,300	Sand and clay	" "	8 "	199	174	5	0
20	500	Clay loam	Moist, direct sun	35 "	5	0	0	0

Early in these experiments it was noted that there occurred a remarkable reduction from the original number of larvae placed in the infestation centers, even after a week's time. It will be noted, from Table 1, that this reduction increased with the number of days

the larvae remained in the soil. Here, as well as in other experiments made, a large percentage of the larvae recovered had lost their sheaths.

This experimental evidence on the failure of the hookworm larvae to migrate in the soil is supported by similar findings of Cort and Payne (1922a, p. 137), in their study on the sources of hookworm infestation in a cane field. Instead of finding a uniform distribution of the larvae over the total area studied, they were found to be definitely localized at the places of soil pollution. Often where two soil samples were taken close together one would contain a large number of larvae, while the other would contain only a few or be even negative. As a specific example, approximately 1,000 hookworm larvae were isolated from a pint of soil taken from a pollution spot 15 yards into the cane. Pint samples taken from the areas within a radius of $1\frac{1}{2}$ feet to 2 yards from this yielded from 0 to 53 hookworm larvae per sample. The environment here was seemingly in every way favorable for migration, yet during the entire time of the investigation, the larvae were always found at more or less definitely localized spots, at the places of soil pollution. That there occurs little or no migration of the hookworm larvae even from centers of very heavy soil infestation has also been shown by Cort and Payne (1922 b, p. 159) under conditions existing in a cacao grove. The evidence from these field studies and the data from the laboratory

experiments given above show that it will be necessary to revise our present conception of the extent of the migrations of infective hookworm larvae, and make it seem very probable that it is only by mechanical means that hookworm larvae can spread from the place of their development.

When Places of Soil Infestation become Dry, Do Hookworm Larvae Migrate to More Favorable Situations ?

To test this point, a large number of hookworm larvae were placed on a small spot in a 13-inch dripping pan, containing moist sand 1 cm. in depth. A second area, to which no larvae were added, was located 8 inches away from the first. The whole surface was then evenly sprinkled with water. Water was afterwards added daily to the second area which alone was kept constantly moistened. Ten days later all the sand appeared to be thoroughly dry except that in and around the watered area. This was transferred to a unit of the small isolating apparatus, and upon examination, proved to be negative for hookworm larvae. A small amount of soil taken directly from the original infestation spot was placed on a glass slide, a few drops of water were added, and upon microscopial examination three shrivelled, completely disorganized hookworm larvae were found. The remaining soil of this area was then examined,but yielded no larvae. This experiment

indicates that infective hookworm larvae cannot migrate from a drying area to a moist area nearby, but, of course, needs confirmation by a larger series of experiments.

Experiments on Passive Migration.

In two experiments made in the same manner as those summarized in Table 1, except that water was added until the surface of the soils was covered, a fairly uniform distribution of the larvae was found in all zones. In the first, with clay loam soil, where 500 hookworm larvae were added to the infestation center, 72 larvae were recovered from that area, 70 from the second, 17 from the third and 3 from the fourth, eight days after the experiment was started. In the second, with a sand and clay soil, 1,300 larvae were placed in the center of infestation. Eight days later 83 larvae were recovered from Zone 1, 271 from Zone 2, 245 from Zone 3 and 258 from Zone 4.

As these two above experiments differed from those of Table 1 only in the amount of water added, it seemed that in these cases the larvae might have been mechanically carried out of the infestation centers by the surface water. In fact, in the examination of preparations in the counting of larvae, they were often found on the surface film.

To determine the numbers of larvae that might be carried from a given center by this means, the following experiments (see Table 2)

TABLE 2

Showing number of hookworm larvae carried in surface film.

Exp. No.	No. larvae used.	Position of larvae in soil.	No. larvae recovered.	Percentage recovery.
1	222	No larvae visible.....	52	23.4
2	234	No larvae visible.....	61	26.1
3	100	No larvae visible.....	28	28.0
4	268	Larvae protruding from surface...	117	43.6
5	127	Larvae protruding from surface...	38	29.9
6	156	Larvae protruding from surface...	57	36.5

were made. Two hundred and twenty-two active hookworm larvae were placed in the center of a 6-inch pudding pan, containing one inch of clay loam soil. The larvae remained undisturbed for several minutes when water was added, until the soil was well covered. The standing water was immediately drawn off with a pipette from all parts of the surface, except that in which the larvae had been placed. This was centrifugated and upon examination of the residue 52 hookworm larvae were found. This showed that 23.4 per cent. of the total number of larvae, placed in the infestation center, had been carried out by the water. Experiments 2 and 3 of this table were made under the same conditions as the first, and their results further illustrate the role water plays in the dissemination of hookworm larvae.

In the first three experiments of Table 2 no larvae were visible on the surface of the soil, at the time the water was added. In experiments 4,5 and 6 of the same table, the larvae had been placed in the soil 15 hours before the water was added. During that time they had established themselves on the surface of the soil, and were found protruding from the prominent soil particles within the infested area. Water was added as before, until the soil was well covered, and upon examination of the residue from the free water slightly higher percentages of the larvae were recovered than in the first three experiments (see Table 2). Apparently when the larvae are well established on the surface of the soil, they are more easily transferred to the surface film.

That hookworm larvae are carried away from centers of soil infestation by the washing of rains, under natural conditions, is indicated by the findings of Cort and Payne (1922, p. 141). Soil samples examined from the drains in the heavily infested area of the cane field studied, showed that the larvae were fairly evenly distributed for a distance of 50 yards. Soil pollution was found only in the higher parts of the drains and their environs. The authors consider that this distribution in the drains is due to the larvae being washed down from the upper ends of the drains, as well as from the areas drained.

To determine the number of hookworm larvae carried on the feet of man, after passage through areas of heavy soil infestation, the

soil was scraped from the shoes of three persons, after they had completed a pollution survey in a cane field (see Cort and Payne, 1922a, p. 115). Soil obtained in this way was examined for hookworm larvae by the routine method, on six different occasions. The findings varied from 2 to 344 hookworm larvae per person. No appreciable difference was noted in the number of larvae obtained, whether rain had fallen twenty-four hours previous to the survey or not. This is explained by the fact that although the amount of rainfall varied, the soil in the cane was always moist enough to cling to the shoes in considerable amounts. The finding of 344 hookworm larvae in the soil adhering to the shoes of one person is significant. This person had undoubtedly stepped in a "nest" of larvae which was immediately transferred to his shoe. Had he been barefoot and the mud remained on the skin, the resulting infestation would probably have been heavy from this one exposure. Again, had the soil been washed off or dropped on a frequented path, there would have been danger to others.

Domestic animals passing through infested areas likewise enter as factors in the dissemination of hookworm larvae. No actual experiments were made to determine this point, but, from observations made, both in cacao groves and cane fields, it seemed evident that a considerable amount of infested soil may be scattered about in this fashion.

The results of my experiments, as well as field observations, have shown that the hookworm larvae are not migratory during their free life in the soil of Trinidad, but that they can be spread to some extent mechanically. Further investigations are needed to test out, under a variety of conditions, the points brought out in this study.

SUMMARY.

1. Experiments carried on in Trinidad, British West Indies, from May to September, 1922, showed that infective hookworm larvae placed on moist soils do not migrate in periods from 15 hours to 42 days.

2. Experiments show that hookworm larvae may be carried out from centers of soil infestation by surface water, and that they can establish themselves in the new locations when the water recedes.

3. Hookworm larvae were not found to migrate to favorable situations when their original environments become unfavorable.

4. Soil scraped from the shoes of men passing through infested areas was found to contain hookworm larvae. It is possible that paths leading to such areas, as well as the immediate surroundings of dwellings, become centers of infestation by the dropping of such soil.

5. During the time the larvae remained in the soil there occured a reduction in their numbers, which increased with the number of days the experiment lasted.

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II. ON THE POSITION OF THE INFECTIVE HOOKWORM LARVAE IN THE SOIL.

While it is known that the soil is the natural habitat of the infective hookworm larvae, only a few observations have been made as to their activities and position in it. Baermann (1917, p. 622), in his studies of the conditions of development and existence of hookworm larvae in the soil, found them to be rather evenly distributed throughout the soil to a depth of 24 cm. He did not find them directly on the surface but close to it. In other experiments, where larvae were studied in soil overgrown with vegetation, he found hookworm larvae constantly in the soil, but never on the plants themselves. No information is given on the exact conditions existing at the time his observations were made. The data from a series of experiments performed by Dr. James E. Ackert, to study the conditions under which hookworm eggs develop and hatch, show that by far the greater number of the hookworm larvae which developed were in the upper half inch of the soil. The complete discussion of these experiments will appear in a later number of this series. Looss (1905, English translation given in Looss, 1911, pp. 421-422) observed mature Ankylostoma larvae on the surface of charcoal cultures when under a high temperature and in a moist atmosphere. He found formations resembling short fungal hyphae, welded together, which upon examination proved to be hookworm larvae. He further observed that the larvae

were not present on the surface when it became drier, but upon addition of water would reappear in 20 to 30 seconds. Smith (1905, p. 192) records the same position of the hookworm larvae on the surface of feces, when exposed to the air for a few days under favorable conditions of moisture. The Georgia State Board of Health (1910) makes the statement that during a rain, or when dew drops collect, hookworm larvae find their way into the water where they remain, if the water does not dry up, until they have an opportunity to penetrate the human body. And when the dew drops dry or the pool of water evaporates the larvae burrow into the moist earth, there remaining until a fresh downpour of rain brings them to the surface again. Dock and Bass (1910) in their book on hookworm disease say that the larvae may crawl up the leaves, stalks or fruit of vegetables. No statement is made of exact observations of larvae doing this. Schultz (1912) found that in cultures from which blades of grass, small sticks or other objects profect, upon which a film of water condenses, hookworm larvae leave the cultures, follow the water film and ascend to the most advantageous point for contact with an eventual host.

Early in the experiments performed in Trinidad, British West Indies, on the migration of hookworm larvae in the moist loam soils (Augustine, 1922) I found them on the surface extending from

the larger and more prominent soil particles. The greater number appeared singly like the hairs on young plants, while others were massed together in flame-shaped formations. Upon close examination with a hand lens the larvae seemed to be in constant undulating movement which greatly increased when the slightest stimulus was applied. A portion of the soil upon which several larvae were extended was placed on a glass slide and treated with a few drops of water. Upon microscopical examination both sheathed and unsheathed larvae were found which left the soil and moved with great rapidity to the margins of the preparation. The bit of soil was then broken and spread over the slide. In it several cast-off hookworm sheaths were found. The larvae on the surface of the soil readily transferred themselves to most objects that touched them, but not to insects, such as ants, as well as small Arachnids which passed through and about the infested centers.

Larvae were found on the surface of the soil in all my experiments, as long as their numbers were sufficiently large for observation and the soil was kept moist. In an experiment to test the effect of direct sunlight on the migrations of hookworm larvae (Augustine, 1922, p. 165) they were found extended from the soil particles as long as the soil remained moist, even at a temperature of 101° F. and in direct sunlight. As the surface of the soil became drier they left their more prominent positions and were found in

the deeper cracks and crevices, disappearing altogether if the drying continued. If water was then added the hookworm larvae reappeared in a few seconds in their previous positions. This process was repeated several times a day with the same result, until their numbers became so reduced that the reactions could not be observed. It seems clear from this experiment that sunlight in itself has no effect upon the movements of the larvae, but influences them indirectly by raising the temperature and increasing the rate of evaporation.

In two experiments on the migration of hookworm larvae (Augustine, 1922, p. 168), where water covered the soil, hookworm larvae appeared extended from short sticks, pebbles and larger soil particles which protruded above the water. They were usually motionless, but the application of a slight stimulus, such as blowing over the surface or jarring the pan, would start them to intense activity, especially where they had formed themselves into petal-like clumps. Toothpicks were placed upright in the soil in the infestation centers. A few hours later the larvae were found one third of the distance up the toothpick, but only as far as the moisture had ascended.

During the study of the active migrations of hookworm larvae in grass-covered soil (Augustine, 1922, p. 165), observations were made to discover whether the larvae would climb upon the blades of grass and collect in drops of water, as has been suggested might happen in

morning dew. With a hand lens larvae were found at the bases of the stems and on the soil within the centers of infestation. They were also found along the outer dead sheaths of the grass which were moist, but never on the green stalks and blades. The green stalks and blades were cut from and surrounding the infestation centers and washed well in water. This water was then centrifugated and the residue examined with the microscope. No hookworm larvae were found. This test was repeated several times with constantly negative results. The probable reason for the larvae not creeping up the green stalks and into drops of water collected in the axiles of the leaves is that there was no continuous film of moisture present as on the dead stalks and sticks. Their movements seem to be definitely limited by the extent of the moisture film.

In connection with a study carried on by Cort and Payne (1922, p. 149) to determine the sources of infestation with hookworm on a cacao estate an attempt was made to duplicate in the laboratory the conditions existing in this environment, to determine the activities of the infective hookworm larvae under such conditions. In a particular grove of this estate the subsoil was a fine, compact, yellow clay. The surface soil contained an abundance of humus and was of a loose texture and dark. Scattered over much of the surface was a layer of decaying twigs and cacao leaves which during the rainy season were constantly moistened byintermittent showers. Here and there the surface of the yellow clay was exposed, and in other places the humus was free from

leaves and twigs. Soil pollution and infestation was found on all three types of surfaces. The soils, twigs and leaves taken for the laboratory experiment were selected from places widely separated from polluted areas. The yellow subsoil was first placed to a depth of $1\frac{1}{4}$ inches in a circular pan, 3 feet in diameter. Two thirds of its surface was then covered with the humus, and half of the humus area was carpeted with the leaves and twigs. At the center of each of these surfaces were placed approximately 1000 hookworm larvae, their positions being marked by circles of cord. On the following day a careful examination was made with a hand lens. Numerous larvae were found extended from the soil particles with in the infestation centers on the yellow clay and humus soils. The larvae which had been placed upon the leaves were not confined within the center, but were found distributed within a radius of eight inches. They were found on the center margins and prominent veins of the leaves and on decaying bark of twigs, especially where the bark was broken and distinct edges were formed. It is probable that the greater extent of distribution of the larvae on the leaves was not due entirely to their active migration but was aided by the washing of the water during the process of moistening; for while the water which was added was immediately absorbed by the two soils, it ran off the leaves, from one to another. Larvae were found within the infestation centers of

of the two soil surfaces at all times until their numbers became greatly depleted. The leaves lost their moisture quickly, and when dry the larvae disappeared to the deeper moisture places, returning to their former position at the next watering.

The position of the hookworm larvae, as found in these and other experiments, is significant since it makes easy the transfer of the larvae from the soil and other infested objects to the human foot.

SUMMARY.

1. Infective hookworm larvae under optimum conditions of moisture and temperature were found to remain on and within the upper surface of the soil.

2. They creep up pieces of wood, decaying vegetation and other objects only as far as the film of moisture extends.

3. Hookworm larvae were not found within the drops of water collected in the axile of the leaves of green plants not upon the leaves themselves.

4. At centers of soil infestation, where the surface is covered with leaves or twigs, the infective hookworm larvae were found extended from the leaves or twigs when moist, but in the soil beneath when dry.

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III. EXPERIMENTS ON THE LENGTH OF LIFE OF INFECTIVE HOOKWORM LARVAE IN SOILS.

Historical Account.

The conception of the length of life of the infective hookworm larvae in the soil which is widely accepted can be illustrated by a quotation from Nicoll (1917, p. 163) : "These larvae (hookworm), when they reach this final stage have ceased to feed, and they can remain alive for months and even years under suitable conditions, i.e., where there is a sufficiency of moisture and not too great heat. In the laboratory they have been kept alive for over 18 months in plain water, at a temperature of about 60 ° F. It can hardly be doubted that they will live fully as long under natural conditions, unless it be that they are attacked and devoured by other animals, such as aquatic insects." The evidence which points to an extended period of life for the mature hookworm larvae comes chiefly from the work of the first seven investigators listed in Table 1. These observations (see Table 1) were made upon sheathed larvae, kept in moist feces or water under laboratory conditions in the temperate

zone. Further, the inference has often been drawn, as in the above quotation, that if the larvae can remain alive for such periods of time under unnatural surroundings, their span of life would be as great or even greater, when in their natural environment.

The work of the last three investigators summarized in Table 1 needs further discussion, since their observations were made under natural conditions. Leichenstern (1887, p. 669) found the larvae of Ancylostoma duodenale alive in the fecal mass from a brick-yard latrine three months after the laborers had left the field. Stiles and Gardner (1910) found two motionless but well-preserved sheathed hookworm larvae in sand 151 days after the sample had been taken from under a surface privy. In experiments by these investigators, where fecal material containing hookworm eggs was covered with sterilized sand, examinations made four and five months later disclosed no hookworm larvae. These experiments were carried on at unheated room temperature in the early fall at the United States Marine Hospital Station, Wilmington, North Carolina. Baermann (1917, p. 622) in his studies on the conditions of development and existence of hookworm larvae in the tropical climate of Sumatra found that the length of life varied under different conditions of moisture and shade. In one box, where a stool containing hookworm eggs was covered with earth by a

TABLE I.

Showing results of previous authors' observations on length of life of hookworm larvae.

Authors	Medium	Environmental conditions	Duration of life
Leichenstern, 1887	Water Moist feces	Laboratory temperature in Germany	7 months
Galli-Valerio, 1905	Moist feces	Laboratory temperature in Switzerland	14 months
Boycott, 1905.....	Moist feces	Laboratory temperature in London, England	More than 12 months
Loebker and Bruns, 1906.....	Water	By laboratory windows Germany	12 months
Oliver, 1910.....	Water	Laboratory temperature England	15 months
Perroncito, 1913...	Water	Laboratory temperature Italy	8-10 months
Nicoll, 1917.....	Water	60° F.	18 months
Leichenstern, 1887.	Fecal material	Abandoned latrine in brickyard, Germany	3 months
Stiles and Gardner, 1910.....	Sand	(Unheated laboratory during early fall, Wilmington, N. C.)	A few alive up to 4 months
Baermann, 1917.....	Soil covered with sod	In the open shade, Sumatra	6 months

a sexton beetle and exposed to sun and rain without additional moistening, some slight development took place, but the larvae were found to have died after three weeks. On shaded, sprinkled

soil the larvae developed in large numbers, lived for 3 months and then died. In boxes planted with grass extensive development of larvae took place in both sun and shade. He found numerous larvae in the unshaded soil up to three months, and a few up to six months in the shaded. The results of these experiments on the length of life of hookworm larvae in the soil, with conditions as nearly natural as possible, gave a shorter period of life than the others in which the conditions were more unnatural, and suggest that soil does not remain infective as long as has been generally believed.

Leuckart (1868, p. 436), many years ago, pointed out that the mature larvae of Ancylostoma canninum (Dochmius trigonocephalus) live at the expense of reserve food stored in the cells of the intestine during development, and as time passes these cells gradually loose their granules, until the worms become transparent. Looss (1911, p. 419) concluded that if the granules deposited in the body are reserve food stores, which are gradually reabsorbed during the later life of the larvae, a limit is set to the length of life, and death will come when this supply is exhausted. He believed that the length of life would depend on whether the supply of food was consumed slowly or rapidly, and since temperature influences to a high degree the motility of the mature larvae, they would remain alive longer at a low than a high temperature. If we accept this view

we should expect the life of the larvae to be the shortest under those conditions of temperature which are most favorable to intense activity, as in the tropics. The results of my experiments carried on in Trinidad, British West Indies, and the field studies of Cort and Payne (1922a and b) support this view.

Material and Methods.

The hookworm larvae used in the experiments, which will be described in this paper, were taken from cultures five to eight days old, with the exception of those used in the series on the length of life of unsheathed larvae which were from an eighteen-day-old culture. The same technique was employed in counting and in preparing the soils as in the migration experiments previously described (Augustine, 1922a). Two sizes of containers were used, viz., (1) tin pudding pans, six inches in diameter and two inches in depth, and (2) three-ounce glass jars.

Data on Length of Life from Migration Experiments.

Early in my work on the migration of hookworm larvae in soils (Augustine, 1922a) I was struck by the great reduction in the numbers of larvae recovered from experiments which had run a few weeks. Table 2 gives the data on the reduction of hookworm larvae in ten of

TABLE 2.

Showing reduction in the numbers of hookworm larvae in the migration experiments.

No. Exp,	No. larvae placed in soil	Type of soil	Time larvae remained in soil	Larvae recovered		
				Un-Sheathed	Sheathed	Total
1	1,000	Moist clay loam	7 days	141	180	321
2	498	Moist sand	10 "	81	10	91
3	512	Moist clay loam	11 "	234	3	237
4	1,100	Moist clay loam	21 "	11	7	18
5	392	" " sod	21 "	1	4	5
6	500	Moist red clay loam	22 "	6	3	9
7	361	Moist clay loam sod	30 "	1	1	2
8	500	Moist clay loam	35 "	2	3	5
9	426	Moist clay loam	37 "	0	1	1
10	650	Moist clay loam	42 "	0	1	1

these experiments. Although the experiments were made under favorable conditions, after a period of twenty-one days, there occurred a reduction in the numbers of larvae of over 90 per cent. Stimulated by these findings, experiments were planned to further test this point.

Experiments on Length of Life of Mature Hookworm Larvae in Soils.

Table 3 gives data from a series of thirty experiments to determine the amount of mass reduction in a known number of mature hookworm larvae in three types of soil, viz., (1) clay loam, (2) sand, and (3) a clay loam sod, under favorable conditions of temperature and moisture. In these experiments the soil was placed to a depth of $1\frac{1}{2}$ inches in thirty of the six-inch pans, ten pans with each type of soil. Three hundred active, sheathed hookworm larvae from cultures five and seven days old were added to each pan. The pans were then placed in a shady place and the soil kept moist throughout the experiment. Examinations, by the isolation apparatus, of the soil from one pan of each type were made weekly for seven weeks and the larvae recovered counted. As the seventh examination gave negative findings for hookworm larvae the eighth examination was made three days later. As this also proved to be negative for all three soils the experiment was closed. The results of these experiments (Table 3) indicate a rapid dying off of the larvae within the first two weeks of the experiment. If the time of development in the cultures is added, it is seen that in three weeks after the eggs left the host 90 per cent of the larvae which developed to the infective stage had perished. The significant points brought out in this series (Table 3) are (1) the high reduction occurring within two or three weeks, (2) the fairly constant number of larvae remaining alive up to seven weeks, and (3) a complete dying out after that time.

It would, therefore, appear that the activities of the larvae are at first intense, which is followed almost immediately by a high death rate.

Table III.

Showing mass reduction of hookworm larvae in 3 types of soil. In each of the experiments 300 sheathed hookworm larvae taken from cultures 5 to 7 days old were placed in 6-inch pudding pans containing $1\frac{1}{2}$ inches of soil. Experiments started July 5, 1921.

Exp. No.	Date of examination.	Clay loam soil. Larvae recovered. Unsheathed	Clay loam soil. Larvae recovered. Sheathed	Clay loam soil. Total	Clay loam soil. Per cent. reduction.	Sandy soil. Larvae recovered. Unsheathed	Sandy soil. Larvae recovered. Sheathed	Sandy soil. Total	Sandy soil. Per cent. reduction.	Clay loam scd. Larvae recovered. Unsheathed	Clay loam scd. Larvae recovered. Sheathed	Clay loam scd. Total	Clay loam scd. Per cent. reduction.
1	July 12	52	4	56	81.3	123	9	132	56	30	9	39	87
2	July 19	16	1	17	94.3	38	1	39	87	10	2	12	96
3	July 26	14	5	19	93.6	13	0	13	95.6	15	3	18	94
4	August 2	13	5	18	94.	11	1	12	96	0	0	0	100
5	August 9	5	0	5	98.3	6	8	14	95.3	6	8	14	95.3
6	August 16	1	14	15	95.	4	0	4	98.6	2	7	9	97
7	August 23	0	0	0	100.	0	0	0	100.	0	0	0	100
8	August 25	0	0	0	100.	0	0	0	100.	0	0	0	100
9 and 10	Not examined												

Table 4 gives the results of a series of experiments similar to those summarized in Table 3, except that the examinations were made every third day and the containers were the three-ounce jars. As the data show, the greatest reduction occurred about two weeks after the experiment was started, or about three weeks after the deposition of the eggs.

The Relation of Loss of Sheath to Length of Life.

As has been previously stated (Cort, Augustine, et al., 1922) a large proportion of the mature hookworm larvae lose their sheaths in the soil. This discovery immediately introduces a new factor into the study of the length of life of the infective hookworm larvae. Three questions present themselves, viz., (1) Does the loss of the protective sheath directly shorten the life of the larvae ? (2) Does the loss of sheath make the larvae more susceptible to unfavorable conditions of environment and thus shorten their life under certain conditions ? and (3) Does the loss of sheath render the larvae non-infective, which would be the equivalent of terminating their lives ?

Table 5 gives the data on a series of experiments on the length of life of unsheathed hookworm larvae in clay loam under favorable conditions. The larvae used in these experiments were isolated from soil on which a stool containing hookworm eggs had been placed eighteen days before. Of the 1,300 larvae obtained only 10 were found enclosed in sheaths. The experiments were made under the same environment

Table IV.

Showing mass reduction of hookworm larvae in a clay loam and sandy soil. In all these experiments the 3 oz. glass jars were used filled $\frac{3}{4}$ full with soil.

Exp. No.	All experiments started July 19	Clay loam soil.					Sand.				
	Date of examination	Number of larvae placed in soil.	Larvae recovered.		Total.	Per cent. reduction.	Number of larvae placed in soil.	Larvae recovered.		Total.	Per cent. reduction.
			Unsheathed.	Sheathed.				Unsheathed.	Sheathed.		
1	July 22	300	128	161	289	3.6	300	83	159	242	19.3
2	July 25	300	115	144	259	13.6	311	99	96	195	37.2
3	July 28	300	69	54	123	59.0	321	90	67	157	51.0
4	July 31	300	42	29	71	76.3	308	107	70	177	42.5
5	Aug. 3	300	59	29	88	70.6	303	29	9	38	87.4
6	Aug. 6	300	33	12	45	85.0	316	48	3	51	83.8
7	Aug. 9	300	40	7	47	84.3	304	40	15	55	81.9
8	Aug. 12	300	5	0	5	98.3	331	38	0	38	88.8
9	Aug. 15	300	13	5	18	94.0	325	26	1	27	91.6
10	Aug. 18	300	3	0	3	99.0	304	21	1	22	92.1

TABLE 5.

On the mass reduction of hookworm larvae in loam soil from cane field.

Ten experiments started July 18, with 100 unsheathed larvae in each. All larvae were obtained from same culture, 18 days old.

Experiment number.	Date of examination.	Number of larvae recovered	Per cent reduction.
1	July 21	87	13
2	24	52	48
3	27	51	49
4	30	15	85
5	August 2	13	87
6	5	4	96
7	8	7	93
8	11	0	..
9	12	0	..
10	12	0	..

as those with the sheathed forms. Examinations were made every third day as long as the results were positive. After the first negative appeared, which was 24 days after the experiments were started, or 42 days after the stool had been placed on the soil, the remaining jars were examined on the day following. These were likewise negative. While this series of experiments indicates that the life of the un-

sheathed hookworm larvae may be similar to that of the sheathed forms it must be taken into consideration that there undoubtedly had occurred a great reduction in the numbers of larvae before isolating them from the culture, and that the larvae used in the experiment were the survivors of this reduction.

While no experiments were made to test the comparative resistance of the unsheathed and sheathed larvae, it was found that upon addition of a few drops of dilute formalin (See Cort, Ackert, et al., 1922, p. 5) to the preparation before counting, the unsheathed larvae quickly succumbed, while the sheathed forms were stimulated to greater activity, and could resist the formalin for a considerable time. This observation indicates, as would be naturally inferred, that unsheathed larvae are less resistant to unfavorable conditions than sheathed. This would then lead to a more rapid dying the unsheathed larvae whenever conditions became unfavorable. Since our present knowledge of the resistance of the mature hookworm larvae to various conditions is based entirely upon studies of the sheathed forms it will be necessary to repeat much of this work with the unsheathed larvae, to determine what actually happens under natural conditions.

That sheathed hookworm larvae complete their second ecdysis, while penetrating the skin, and become adults in the intestines of man has been conclusively demonstrated by a number of investigators. Whether the loosing of the sheath in the soil renders the larvae non-

infective, as far as man is concerned, which would be equivalent to their death, is not yet known. To gain information on this point unsheathed larvae were applied to the shaven belly of a male white rat, sixteen months old. Upon examination with a binocular microscope the larvae were seen to quickly spread themselves over the surface of the skin as far as the moisture extended. As the moisture around the larvae evaporated penetrating movements were started. The anterior ends of the worms would be lifted into the air and then turned toward the skin again, touching here and there as if seeking some port of entry. In one particular instance a larva was seen to leave the shaven area, to settle at the base of a hair and push one third of its body into the hair follicle. Then after a moment's rest the posterior end swung up parallel with the hair and with several slow twists the worm moved further into the skin and again rested. This movement was repeated several times until the tail of the worm vanished beneath the surface of the skin. Two other unsheathed larvae were seen to penetrate the skin within the shaven area. The entire process took less than half an hour. One hour later water was applied to the infected spot and upon microscopical examination no larvae were found. While this observation only shows that unsheathed larvae can penetrate mammalian skin, it seems very probable that they can also reach the intestine

and grow to maturity. Further experiments are needed to clear up this point.

Discussion of Results.

This experimental evidence on the length of life of the infective hookworm larvae in the soil is supported by the field observations of Cort and Payne (1922a, p. 138). In one instance soil pollution was found near the edge of a cane field and soil samples from this area showed it to be an intense center of infestation. This area was visited frequently at later intervals, but no further evidence of soil pollution was found. Soil samples, taken three weeks later from the same place, yielded but nine hookworm larvae, and later examinations showed a complete dying out of the larvae. More striking still is their study of the mass reduction of hookworm larvae in a chosen area of this field which, before educational work, sanitation, and treatment were started, was found to have a very heavy infestation with hookworm larvae. In less than three weeks after the reduction of soil pollution, which immediately followed the introudction of control measures, a second series of examinations was made. The results of these examinations showed a reduction of over 90 per cent from the first series. Subsequent examinations of samples taken from this area showed still further reductions. In about six weeks

after pollution of the soil had been stopped this once heavily infested strip of cane field had become practically free from hookworm larvae. Similar results were obtained during their studies on the sources of hookworm infestation in a cacao grove (Cort and Payne, 1922b). Although soil pollution was not checked samples of soil taken from grossly polluted areas, six weeks after treatment, showed a marked reduction in the number of infective hookworm larvae present.

The results of these laboratory experiments and field observations show that the life of the hookworm larvae in the soils of Trinidad is limited to a few weeks. Whether this finding is constant under a variety of conditions is not known, and experiments are planned to test this point. These results are very different from those obtained with sheathed larvae under laboratory conditions at lower temperatures. It would seem that the differences in these results can be explained by differences in the conditions of the experiments. The conditions of the experiments of the early workers (see Table 1), in which larvae were kept alive for such long periods, were such that the activities of the larvae were greatly reduced and the protective sheaths retained. In my experiments in Trinidad the daily range of temperature during the course of the work was from about 74° F. to 94° F., a temperature which, if other conditions

were favorable, tended to keep the larvae active. Further it was found that a large proportion of the larvae lost their sheathes in the soil. While it is not possible from the information at hand to find any direct correlation between the process of unsheathing and the death rate under favorable conditions, it was found that the unsheathed larvae are more susceptible when in unfavorable environments. As the unsheathing of the larvae appears to be a continuous process, as long as the soil remains infective, the newly unsheathed larvae, would be constantly killed when becoming exposed to diverse conditions, even though sheathed larvae were able to withstand the diversity. It can be concluded therefore, that conditions favorable to intense activity and to the loss of the sheath will tend to shorten the life of mature hookworm larvae in the soil.

SUMMARY.

1. From the observations of various investigators on hookworm larvae under unnatural conditions the opinion has become current that they live for months or even years in the soil, under favorable conditions of temperature and moisture.

2. Laboratory experiments carried on in Trinidad, British West Indies, from May to September, 1921, show that a rapid reduction in the number of hookworm larvae occurs in soils

of various types, and that the extent of their life is limited to about six weeks.

3. The completion of the second ecdysis of the infective hookworm larvae in the soil presents a new factor in determining their length of life, which was not found to directly shorten the life of the larvae under favorable conditions, but renders them more susceptible when in unfavorable environments.

4. Experimental evidence indicates that the loss of sheath does not render the mature larvae non-infective which would be the equivalent of their death.

5. The conclusion can be drawn that environmental conditions, such as tropical temperatures which tend to increase the activity of the mature hookworm larvae, will shorten their lives by the more rapid using up of the stored food material.

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IV. VARIATION IN LENGTH OF LIFE OF HOOKWORM LARVAE FROM THE STOOLS OF DIFFERENT INDIVIDUALS

In connection with studies on the length of life of infective hookworm larvae in soils under varying conditions of environment at Utuado, Porto, during the summer of 1922, I made an experiment to ascertain whether or not a difference existed in the vitality and life span of larvae developing from the stools of different persons. To determine this point fresh stools were obtained from twenty-two infected individuals living in and near Utuado. Two were of the village itself and the remaining twenty were from the surrounding mountains and valleys. This group, which represented fourteen families, included adult field workers, men and women, women housekeepers, children working part time in the fields, women who were no longer able to work in the fields due to severe hookworm infections, carpenters and milk venders. The stools were cultured in soil which had been previously heated to kill all free-living animals present. The hookworm larvae were isolated after seven days' incubation.

The same general technique was employed in this experiment as was used in the experiment on the length of life

of hookworm larvae made in Trinidad, B. W. I. (Augustine, 1922 b). The larvae were counted by a dilution method which was found to insure a surprisingly uniform number of larvae in each container. This method consists in placing an estimated quantity of larvae in a 15 ml. centrifuge tube and adding sufficient water - about 10 ml. was found convenient - so that after thorough mixing by drawing the water in and forcing it out of a small graduated pipette, 0.5 ml. will contain approximately the number of larvae desired for the experiment. That this method is accurate enough to be used in length of life experiments of this type is shown by the results of ten consecutive counts of samples taken from the same suspension. The results of these counts were as follows: 568, 518, 500, 525, 521, 504, 564, 512, 515, and 521. Three accurate counts were made of the larvae in the first three 0.5 ml. amounts for each of the twenty-two series, and the average was used to denote the number of larvae per unit of a series. The reductions, percentages of reductions and the number of larvae remaining alive after an isolation were computed from this number.

The larvae thus counted were placed in three-ounce glass jars filled three-fourths full

of light, moist, sandy loam soil, of a type common throughout the Utuado district. Ten such jars were prepared for each individual. In all, twenty-two series of ten units each constituted the experiment. The jars were placed on a table on the east gallery of the laboratory, where they were protected from rain and direct sunlight by the gallery roof and nearby palm trees. They were, however, subjected to the drying winds and diffused sunlight played upon them for a short time in the morning.

The surface of the soil in these jars was allowed to become dry each time before more water was added. The soils required moisture about every two or three days. Such an environment for this experiment was chosen for two reasons, viz., (1) to test the length of life of hookworm larvae in an alternating moist and drying soil, and (2) to test in an environment which is somewhat unfavorable, the resistance and length of life of infective hookworm larvae from the stools of different individuals. Examinations were made weekly by isolating the larvae from the soil with the isolation apparatus described in number II of this series (Cort, Ackert, et al., 1922). Wherever a unit was found to be negative, another unit of the same series was examined

one or two days later. If this unit was also negative that series was closed and the length of life of the larvae was regarded as having terminated at the time of the first negative. However, if larvae were isolated after the first negative, the examination of the series was continued at weekly intervals until two successive negatives were obtained.

Table I gives a summary of the twenty-two experiments showing the number of larvae recovered, with the percentages of reduction occurring on the dates of examination. Chart 1 shows graphically the differences in the rate of reduction in eight of the experiments from Table I. The number above each curve corresponds to the series number of Table I. The rate of mortality in this experiment is higher than the rates for larvae kept in the same environment, but with the soil constantly moist. This higher death rate can be attributed to the alternating moist and drying condition of the soil. The significance of this difference will be discussed in a later number of this series.

Chart 2 is constructed from the data in Table I and represents the limits in length of life of the infective larvae for each of the individuals. It strikingly illustrates the variations in the length of life of hookworm larvae obtained

TABLE I.

Showing reduction in numbers of hookworm larvae from the feces of twenty-two individuals, in an alternating moist and drying soil.

Series Number.	Number larvae per unit.	Date series was started.	Dates of examinations of units.								
				7/25	8/1	8/8	8/15	8/22	8/29	9/5	9/12	9/14
1	415	July 18	Larvae recovered	202	112	32	8	21	20	1	0	0
			Percentage reduction	51.3	73.0	92.2	98.0	94.9	95.0	99.8	100	100
				Dates of examinations of units.								
				7/27	8/3	8/10	8/17	8/24	8/31	9/7	9/14	9/16
2	605	July 20	Larvae recovered	39	51	21	18	7	2	2	0	0
			Percentage reduction	93.5	91.5	96.5	97.0	98.8	99.6	99.6	100	100
				Dates of examinations of units.								
				7/27	8/3	8/10	8/17	8/24	8/31	9/1	9/7	9/8
3	448	July 20	Larvae recovered	44	41	14	7	1	0	2	0	0
			Percentage reduction	90.1	90.8	96.8	98.4	99.7	100	99.5	100	100
				Dates of examinations of units.								
				*8/3	8/10	8/17	8/24	8/31	9/7	9/14	9/16	
4	283	July 27	Larvae recovered	234	92	65	14	8	2	0	0	
			Percentage reduction	17.3	67.4	77.0	95.0	97.1	99.2	100	100	
				Dates of examinations of units.								
				8/5	8/12	8/19	8/26	9/2	9/9	9/16	9/18	
5	691	July 29	Larvae recovered	72	53	61	8	2	1	0	0	
			Percentage reduction	89.5	92.3	91.1	98.8	99.7	99.8	100	100	

TABLE I. (*Continued*)

Series Number.	Number larvæ per unit.	Date series was started.		Dates of examinations of units.								
				8/7	8/14	8/21	8/28	9/4	9/11	9/18	9/19	
6	584	July 31	Larvæ recovered	94	48	12	4	2	3	0	0	
			Percentage reduction	83.9	91.7	97.9	99.3	99.8	99.4	100	100	
				Dates of examinations of units.								
				8/3	8/10	8/17	8/24	8/31	9/7	9/9		
7	147	July 27	Larvæ recovered	17	20	7	2	2	0	0		
			Percentage reduction	88.4	86.3	95.2	98.6	98.6	100	100		
				Dates of examinations of units.								
				8/5	8/12	8/19	8/26	9/2	9/9	9/10		
8	343	July 29	Larvæ recovered	34	5	12	5	6	0	0		
			Percentage reduction	90	98.5	96.5	98.5	98.2	100	100		
				Dates of examinations of units.								
				8/13	8/20	8/27	9/3	9/10	9/11			
9	525	Aug. 6	Larvæ recovered	98	33	20	30	0	0			
			Percentage reduction	81.3	93.7	96.1	94.2	100	100			
				Dates of examinations of units.								
				8/9	8/16	8/23	8/30	9/6	9/7			
10	745	Aug. 2	Larvæ recovered	156	12	6	5	0	0			
			Percentage reduction	79.0	98.3	99.1	99.3	100	100			

TABLE I. (*Continued*)

Series Number.	Number larvae per unit.	Date series was started.	Dates of examinations of units.								
				8/9	8/16	8/23	8/30	9/6	9/7		
11	748	Aug. 2	Larvae recovered	64	4	2	1	0	0		
			Percentage reduction	91.4	99.4	99.7	99.8	100	100		
				Dates of examinations of units.							
				8/10	8/17	8/24	8/31	9/1	9/7	9/8	
12	396	Aug. 3	Larvae recovered	10	6	6	0	3	0	0	
			Percentage reduction	97.4	98.4	98.4	100	99.4	100	100	
				Dates of examinations of units.							
				8/10	8/17	8/24	8/31	9/7	9/9		
13	348	Aug. 3	Larvae recovered	14	6	1	2	0	0		
			Percentage reduction	95.9	98.2	99.7	99.4	100	100		
				Dates of examinations of units.							
				8/13	8/20	8/27	9/3	9/4			
14	457	Aug. 6	Larvae recovered	35	4	2	0	0			
			Percentage reduction	92.3	99.1	99.5	100	100			
				Dates of examinations of units.							
				8/4	8/11	8/18	8/25	8/26			
15	650	July 28	Larvae recovered	23	45	5	0	0			
			Percentage reduction	96.4	93.0	99.2	100	100			

TABLE I. (*Continued*)

Series Number.	Number larvae per unit.	Date series was started.		Dates of examinations of units.								
				8/9	8/16	8/23	8/30	8/31				
16	203	Aug. 2	Larvae recovered	25	8	2	0	0				
			Percentage reduction	87.6	96.0	99.0	100	100				
				Dates of examinations of units.								
				8/10	8/17	8/24	8/31	9/1				
17	456	Aug. 3	Larvae recovered	13	3	1	0	0				
			Percentage reduction	97.1	99.3	99.7	100	100				
				Dates of examinations of units.								
				8/11	8/18	8/25	9/1	9/2				
18	528	Aug. 4	Larvae recovered	8	9	1	0	0				
			Percentage reduction	98.4	98.2	99.8	100	100				
				Dates of examinations of units.								
				8/9	8/16	8/23	8/24	8/30	8/31			
19	464	Aug. 2	Larvae recovered	37	3	0	1	0	0			
			Percentage reduction	92.0	99.3	100	99.7	100	100			
				Dates of examinations of units.								
				8/8	8/15	8/22	8/23					
20	344	Aug. 1	Larvae recovered	5	2	0	0					
			Percentage reduction	98.5	99.4	100	100					

TABLE I. (*Continued*)

Series Number.	Number larvae per unit.	Date series was started.		Dates of examinations of units.									
				8/7	8/14	8/21	8/23						
21	512	July 31	Larvae recovered	41	11	0	0						
			Percentage reduction	91.9	97.8	100	100						
				Dates of examinations of units.									
				8/11	8/18	8/25	8/27						
22	433	Aug. 4	Larvae recovered	5	2	0	0						
			Percentage reduction	98.8	99.5	100	100						

CHART 1. Comparison of the rate of mortality at weekly intervals and length of life of hookworm larvae from eight different individuals, in an alternating moist and drying soil.

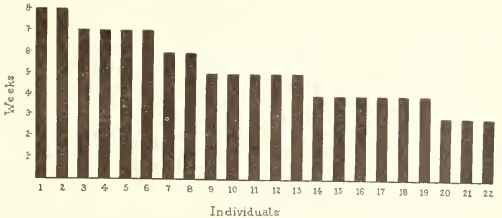

CHART 2. Representing the limits of the life span of the hookworm larvae, from twenty-two different individuals, in an alternating moist and drying soil.

from the different individuals. Each bar represents the time from the isolation of the larvae from the cultures to finding the series negative. The actual dying out of the larvae therefore occurred some time during the last week. It can be seen, therefore, that the hookworm larvae obtained from cultures of the feces of a number of individuals show differences both in the rate of dying out (see chart 1) and in the final limits of their life (see chart 2).

No adequate explanation of this variation in the length of life of hookworm larvae from different individuals can be given from the data obtained from this experiment. Neither could a definite correlation be discovered between the age, food habits, or occupation of the various individuals and the length of life of the larvae coming from them.

This difference in length of life of infective hookworm larvae from different persons may be due to several factors. The length of life of the larvae developing from the eggs of a single female may be an inherited characteristic. Certain races may have a longer span of life than other races. This would, however, only account for a certain number of larvae coming from a single stool, for a given infestation would probably not represent a parasitism on the part of a single race. A few larvae are picked up here and there and at different intervals, so that an infected person would probably harbor many races. It is possible that the diet and physical condition of the

infected individuals affects the physical condition of the adult worms in the intestine, which in turn would influence the character of the eggs and larvae. The character of the diet of the individual may directly affect the length of life of the larvae. Looss (1911, p. 374) found a marked variation in development of the larvae due to the character of his diet. We should expect a longer length of life among well-nourished larvae than among those which have insufficient nutrition. Stoll (1923), in a series of laboratory experiments, found that a poor development of larvae occurred in acid stools from individuals on a high vegetable diet. We should therefore expect a shorter span of life among the larvae developing in an unfavorable environment than in one which is favorable. Again, the excrements from persons ill or physically weak may contain certain excretions from the body in such amounts as to alter the normal length of life of the larvae developing in them.

It is probable that there is not one factor alone responsible for this variation. The important point, however, that that this experiment has shown is that a difference does exist in the larvae obtained from different

individuals. Such variations may in part account for the differences in the results of investigators on the length of life of infective hookworm larvae.

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V. OBSERVATIONS ON THE COMPLETION OF THE SECOND ECDYSIS OF NECATOR AMERICANUS.

Historical Account.

In 1880 Perroncito discovered that upon certain manipulations of a microscopical preparation of mature hookworm larvae, the sheaths would break and the enclosed larvae escape. Leichtenstern (1887) also observed this phenomenon in hookworm cultures four to five weeks old, after the sheath had become brittle, and stated that a new covering was formed a few days later in the place of that cast. He was of the opinion that the casting of the sheath occurred only among old larvae. In 1902 Lambinet succeeded experimentally in inducing infective larvae to unsheath by placing them in gelatine. He observed that in this medium unsheathing was a purely mechanical process, the efforts of the larvae freeing them from their sheaths which were held fast in the medium. Herman (1905) also induced larvae to cast their sheaths by placing them in a small amount of aqueous solution of an aniline dye. Looss (1911), on repeating Herman's experiments, found that the larvae did not become unsheathed in the solution of the dye, regardless of the concentration, except beneath a cover-glass and then only

when a very thin layer of the liquid remained between the slide and the coverglass. Looss also found that infective larvae became unsheathed in water only under these same conditions. From his observations he concluded that foreign particles must be present in the water or the medium be of sufficient viscosity to retain the sheaths so that the larvae can mechanically force their way out. In observing the process of unsheathing in staining fluids Looss found that the movements of the larvae were greatly increased immediately after contact with the stain. When the layer of the fluid was thick the movements of the larvae were of a boring nature, head downward on the slide and never in an upward direction against the coverglass. He is of the opinion, (1911, p. 433), "that the increased stimulation caused by the stain is in some way comparable to the conditions the larvae meet either on or in the body of their host; so that they are led to believe that they have reached their final destination." He considers that the ecdysis in dye solutions is actively produced by the larvae; that is, they exert themselves to leave their envelopes, and under favorable conditions succeed. In preparations made of water only he considers the mode of unsheathing as passive as far as the larvae are concerned and thinks it results from the mechanical retention of the sheath

while the larva breaks its way out by trying to move onwards. Looss concludes, however, that the process of ecdysis itself is purely mechanical, and remains the same whether performed actively or passively by the larvae. The observation by Leichtenstern of the larvae becoming again ensheathed after once casting the old skin was not confirmed by Looss and he clearly shows that such a process is improbable a priori from biological reasons.

It is interesting to note, that although the factors responsible for the unsheathing of mature larvae were observed by so many of these earlier investigators under experimental conditions they were not recognized as operating in the natural environment of the larvae, namely, the soil. It was believed that in the normal course of events the sheath was only thrown off when the larva penetrated the skin of the host and entered the third stage of its life.

In 1917 Baerman noted the presence of unsheathed forms in his isolations of hookworm larvae from samples of soil taken from infested areas. The real significance of this finding was not realized and he attributed the loss of their sheaths to the passage through the apparatus used. That some unsheathing may occur during the passage of larvae through the isolation apparatus was found during our experiments on

the standardization of an apparatus similar to that used by Baerman (Cort, Ackert et al, 1922).

While closely examining the surface of the soil in one of my experiments on the migrations of infective hookworm larvae during the summer of 1921 (Augustine, 1922 a) larvae were found protruding from the more prominent soil particles and were confined within the center of pollution. To determine whether or not the larvae were distributed throughout the soil clumps a small portion was placed on a glass slide, cut in two and a few drops of water added. Upon examination with the microscope three active unsheathed larvae were found. Further observations were made upon soil taken from other experiments and the presence of unsheathed larvae and their cast sheaths were always found. These findings indicated that the completion of the second ecdysis might be a common process under natural conditions and were confirmed both in other laboratory studies and in the field investigations of that summer (Cort, Augustine, et al., 1922) (Cort and Payne, 1922). During the summer of 1922 this phenomenon was constantly encountered both in the laboratory experiments and field studies made at Utuado, Porto Rico.

Comparison of the First and Second Ecdysis of Hookworm Larvae.

A comparison of the development of the hookworm larva from the time it hatches from the egg with the period after the first ecdysis to the infective stage shows certain definite physiological differences which make the two ecdyses distinctly unlike. The first ecdysis is due to the increasing size of the larva which, as it were, bursts the old skin. After the larva has reached maturity, however, it shrinks in size and a space results between the separated cuticula and the larva. This old skin or sheath is then retained and serves as a protective covering unless broken and shed, usually with the aid of outside agencies. The following experiments and observations were made to gain information on the nature of these agencies and their relation to the casting of the sheaths of infective hookworm larvae.

Material and Methods.

The infective larvae of Necator americanus used in the unsheathing experiments were isolated from soil cultures incubated at 27° C. for six days. Under six days many transitional forms were encountered which are undesirable for such studies. The unsheathed larvae recovered at the close of an experiment on the relationship of the coarseness of the soil to

the rate of unsheathing were used in comparing the resistance of the sheathed and unsheathed forms to various chemicals. The same general technique was employed in counting the larvae and preparing the soils in these experiments as has been previously described (Augustine, 1922a). In addition to these experiments direct evidence was obtained regarding process of unsheathing and on the resistance of the sheathed and unsheathed forms during the studies on the length of life of infective hookworm larvae under varying conditions both in Trinidad and Porto Rico. These experiments will be discussed here in this relationship only.

Experiments and Observations on Factors Influencing the Loss of the Sheath.

The experiments on the unsheathing of mature hookworm larvae in water, agueous methyl green and gelatine, as made by Looss, were repeated and my observations confirm his in showing that the ecdysis is a purely mechanical process. It was found however, that the larvae may become unsheathed in water only. This phenomenon had been already observed by Liefmann (1905, p. 357) but not confirmed by Looss (1911, p. 434). In my experiments the unsheathing in water more frequently occurs among older larvae, about eight weeks of age, although it may take place among relatively young mature forms. Under these circumstances

the old skin is broken during the lateral flexures of the larva. This break in the sheath usually occurs a bit anterior to the nerve ring or at the middle portion of the enclosed larva (Fig. 1). It is at these two points that the greatest stress plays upon the sheath during the activities of the larvae and where the well known regular wrinkles of the sheath appear most prominently. The break is always clear cut and apparently follows a striation. It is generally believed that the sheaths of infective larvae become more brittle with age. Accepting this as true, the increased number of empty sheaths among older larvae is accounted for by the increasing rigidity of the sheaths which renders them more breakable during the lateral flexures of the larva.

From my observations of mature hookworm larvae in staining fluids, particularly in aqueous solutions of methyl green, as used by Looss, I cannot attribute the boring movements to the action of the dye. Neither can I liken the properties of the staining solution to those which act upon the larvae on, or in, the body of the host (see Looss, 1911, p. 433). These boring movements of the larvae are also constant in aqueous preparations when examined under the microscope and are apparently due to the light and heat coming up, through the

condenser of the microscope. To test this point larvae were placed on a glass slide and a few drops of aqueous methyl green were added. No cover glass was used. Upon microscopical examination with light obtained from an electrical substage illuminator the larvae were found to be in great activity with heads downward. I then heated the blade of a small knife and placed it to one side of the preparation. The larvae almost immediately turned away from the light coming from below and moved in the direction of the heated blade. When the knife blade was removed the larvae again moved toward the light and resumed their former activities. The knife blade was heated and held directly over the preparation and the light from below was reduced to a minimum. The position of the larvae was now reversed and although they did not reach the upper surface of the preparation their movements were distinctly headed in that direction. The light from below was then removed and the preparation was allowed to remain on the stage about 15 minutes. Upon replacing the light most of the larvae were found to be motionless while others were moving in the horizontal plane. Within a few seconds the active larvae again took upon themselves the boring movements on the substratum and in a few minutes later the inactive ones gave the same response. While it is true that the dye may have stimulated the larvae to

greater activity, a similar reaction occurs in thin water preparations. Here the increased stimulation is apparently due to the pressure of the cover glass upon the larvae or in absence of the cover glass the pressure of the surface film. As to the stimuli responsible for the boring movements of the larvae, as observed under the microscope, heat and light are evidently very important, heat playing a greater role than light. It seems plausible to attribute the heat of the body as being an important factor in directing the larvae within its host. Similar reactions of the larvae to heat have been observed by Khalil, (Khalil, 1922).

The actual casting of the sheath is described by Looss (1911, p. 432) as occurring from the result of a tear in the outer covering constantly taking place in such a way that a cap, about 0.02 mm. long is split off along a line running at right angles to the axis of the body. The margin of the skin rebounds at the same instant almost to the level of the nervous system. The cap was found to remain on the head of the larva for sometime, and when the break was not complete to adhere to the rest of the skin. The further casting of the old skin was found to proceed rapidly and to be accomplished after a few efforts of the larvae.

In my experiments the manner of unsheathing has

not been constant. It seems to have a definite relation to the character of the environment. As has previously been described, the completion of the second ecdysis in water alone takes place by a break in the sheath due to the lateral flexures of the larva. In microscopical slide preparations with water or aqueous dye solutions on which a cover glass is used the casting of the sheath usually results after a longitudinal tear along one of the median lines. This break is brought about by the mechanical retention of the sheath by the slide and cover glass and the force of the larva moving forward, which at first stretches the sheath and later produces the rip. (See fig. 2).

Further experiments were made on the unsheathing of mature larvae in three different types of environments, viz.: (1) moist ground glass, (2) moist sand and (3) a moist garden loam containing a fair amount of decaying vegetation. These substances were placed in syracuse watch glasses and an approximately equal number of sheathed larvae added to each. Fifteen hours later bits of the surface were transferred to glass slides, a few drops of water added and a mocroscopical examination was made of the empty capsules present. The empty sheaths found in the ground glass appear entirely free from clinging debris and

their anterior ends show a clean break either at a right angle with the long axis of the body or at a slighter angle (see fig. 3). Upon isolation of the larvae from the glass after fifteen hours, over 40% were found to be without sheaths. As newly matured larvae do not become unsheathed in water to this extent, at least in 15 hours, the loss of the sheaths in this instance may be attributed to their tips being cut by the sharp edges of the glass during the activities of the larvae.

In sand a large number of larvae become unsheathed due to the wearing away of the anterior end of the sheath against the rough and sharp particles. Here the tip of the sheath presents a jagged appearance and usually but little debris is found clinging to it. (see fig. 4). In the clay loam soil larvae lose their sheaths by first boring between small closely packed masses of soil or into bits of decaying vegetation, (see figs. 5 and 6) which hold the sheaths fast. The sheath is then torn at the anterior end by the larva moving onwards. The process is identical with that observed under the microscope in very thin preparations. From these observations it is apparent that the completion of the second ecdysis of infective larvae may be accomplished in different ways depending largely upon the character of the environment.

Table I gives the data obtained from a series

of experiments to determine the relation of the amount of unsheathing to the size of the particles of the medium. In these experiments sand was graded by sifting it through a nest of five sieves of 20, 40, 60, 80 and 100 mesh respectively. The graded sand was placed in syracuse watch glasses and moistened after the addition of the larvae. The experiment when set consisted of 14 units; the units each of the whole sand, two units each of the grades retained by the 20, 40, 60, 80 and 100 mesh sieves and two of the grade passed through the last sieve. Isolations of a unit of each grade were made 15 hours after the experiment was started. The second set of units were examined one week later. The soils were kept constantly moist during this period.

In both isolations however, percentages of unsheathed larvae were recovered in the coarser grades. With the increasing fineness of the soil particles this percentage increases until a grade is reached where the number of unsheathed forms decreased. These results seem to indicate that in the coarser soils the larvae though free to move do not meet the obstacles which hold the sheaths as in the finer grades and that in extremely fine soils they are unable to move through the soil to any extent and therefore less unsheathing occurs. The results obtained from

Table 1. Showing extent of unsheathing of mature hookworm larvae in graded soils.

No.	Grade of soil	1st Series				2nd Series			
		Per cent recovered after 15 hours		Per cent recovered after 1 week		Per cent recovered after 15 hours		Per cent recovered after 1 week	
		Sheathed	Unsheathed	Sheathed	Unsheathed	Sheathed	Unsheathed	Sheathed	Unsheathed
1	Whole sand	14.5	85.5	27.3	72.7	41.6	58.4	2.5	97.5
2	Retained by 20 mesh sieve	50.0	50.0	54.6	45.4	74.0	36.0	16.7	83.3
3	Retained by 40 mesh sieve	34.5	65.5	19.2	80.8	54.8	45.2	22.0	78.0
4	Retained by 60 mesh sieve	17.7	82.3	7.7	92.3	28.3	71.7	23.7	76.3
5	Retained by 80 mesh sieve	14.5	85.5	--	100.	16.1	83.9	11.2	88.8
6	Retained by 100 mesh sieve	10.0	90.0	2.1	97.9	37.2	62.8	23.1	76.9
7	Passed through 100 mesh sieve	25.0	75.0	21.5	78.5	57.2	42.8	20.0	80.0

the last examinations of the second series do not conform with those of the first series. It is apparent that some outside factor, which cannot be explained at this time, is responsible for these differences.

Comparative Resistance of Sheath and Unsheathed Larvae.

That unsheathed hookworm larvae are less resistant than the sheathed forms when in unfavorable environments has been previously noted (Augustine, 1922, b). To gain further information on this point a series of observations were made on the reactions and lengths of life of both types in various chemicals. The larvae were placed in hollow ground slides and the chemicals added after the excess water was removed. In a 1% carbolic acid solution both sheathed and unsheathed larvae were quickly stimulated into intense activity. Fifteen minutes after the addition of the acid the unsheathed forms were sluggish and their movements entirely spasmodic. After 22 minutes all of these were dead. The sheathed larvae were extremely active for 20 minutes after which the rate was noticeably reduced. All were dead after 40 minutes and disintegration was observed after 45 minutes. None of the larvae regained activity upon replacing the acid with water. In a 1% formalin solution a few unsheathed larvae lived for 35 minutes while the majority appeared inactive and

and lifeless after 20 minutes. Sheathed larvae in the same solution lived for 55 minutes. Neither forms regained activity when placed in water. In 10% formalin unsheathed larvae died within 3 minutes. Out of a large number of sheathed forms one persisted for 30 minutes while the others showed a greater susceptibility and died within 23 minutes. Similar results were obtained with saturated corrosive sublimate. Many of the unsheathed larvae died within the first 4 minutes after the addition of the sublimate. A few lived for 30 minutes. All the sheathed larvae were active for 30 minutes and died after 65 minutes. Each of the above used chemicals has a characteristic effect on the larvae which aids in determining the time of death. Shortly after a larva has ceased activity in carbolic acid solutions a clear discharge appears at the opening of the buccal cavity. A few minutes later the intestinal cells appear as if "cleared" and the worm lies outstretched (see text fig. 7).

In formalin the larvae coil up immediately upon cessation of the lateral flexures and appear as perfect rings. The intestinal cells remain well preserved and retain their characteristic sheen (see fig. 8). Corrosive sublimate has much the same effect upon the larvae as the formalin except that they usually do not coil but lie stretched out (see fig. 9).

Table II gives further data on the comparative resistance of sheathed and unsheathed hookworm larvae which are taken from one of the Porto Rican experiments on the length of life of the larvae in a drying clay loam soil. The details of this experiment will be published in a later number of this series. Suffice it to say that at the beginning of the experiment the amount of soil and water in each container were uniform (by weight) and that the amourt of water present in the soil was determined on the day of examination. The soils received no additional water after the experiment was begun. The experiment was carried on under dense shade. A study of the percentages of sheathed and unsheathed larvae appearing on the first and following examinations (see Table II) will show (1) that a large number became unsheathed early in the experiment and (2) that the unsheathed forms were the first to succumb to the adverse condition. As the sheathed larvae recovered on the last examinations were well nourished, which was probably due to inactiviity, the mortality can only be attributed to dessication. The above experiments which show distinct differences between sheathed and unsheathed larvae in their resistance to adverse conditions, indicate that the percentage of unsheathed larvae in a given environment must be considered when estimating the effect of adverse con-

Table II. Showing the comparative resistance of sheathed and unsheathed hookworm larvae in a drying clay loam soil under dense shade. The experiment was started June 29, 1922.

No. of unit	No. larvae per unit	Day examined	Larvae Recovered		Total	%Sheathed	% Unsheathed
			Sheathed	Unsheathed			
1	366	July 4	160	134	294	54.5	45.5
2	357	" 9	165	22	187	88.3	11.7
3	390	" 14	201	26	227	88.6	11.4
4	400	" 19	59	3	62	95.2	4.8
5	372	" 24	51	0	51	100	0
6	414	" 31	15	0	15	100	0
7	401	August 7	0	0	0	0	0
8	488	" 8	0	0	0	0	0

ditions on infective hookworm larvae.

Infectivity of the Unsheathed Hookworm Larvae.

It was previously demonstrated that unsheathed hookworm larvae can penetrate mammalian skin (Augustine, 1922, b). The following experiment was made to determine whether they can also reach the intestine and become adults. Twenty-five unsheathed larvae of Necator americanus were placed on the left forearm of a volunteer who had been examined previously at various intervals and found to harbor no intestinal worms. The larvae were obtained from soil cultures 7 days old and were later recovered after losing their sheaths in moist sand in which they had been placed 24 hours previous. After the larvae were applied to the arm a moist gauze bandage was placed over the infected area and fastened with adhesive tape. Within 20 minutes definite irritation was sensed, which continued for five days. The gauze was removed after one hour and 19 distinct salmon red lesions were counted where the larvae had entered the skin. Examinations made by the smear and Kofoid Barber brine loop flotation methods were negative 2 and 4 weeks later but were positive for hookworm ova on the 6th week after the penetration of the skin by the larvae.

From a comparative study, made by N. R. Stoll, of the egg output of the above infection with a parallel infection developing from 50 sheathed larvae in a second individual there appears

to be little or no difference in the infectivity of the two forms. In the first infection, A, with 25 unsheathed larvae an average of about 40,000 eggs per day were expelled on a ten-day study made five months after the skin infection. In infection B, with 50 sheathed larvae an average of about 100,000 eggs per day were expelled on a forty day study made after the same interval of time. While infection A was 50% of B at the time of skin infection its egg count per day shows it to be 40% of B's at the time of the egg counts made 5 months later. The difference is probably insignificant. The details of these observations will be published in a later number of this series.

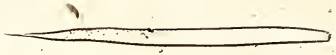

Fig. 1. Sheath cast in water.

Fig. 2. Sheath cast by its retention by the slide and the cover glass and the force of the larva moving forward. The larva escapes by a tear at the anterior end of the sheath.

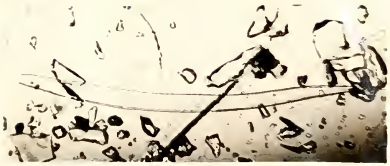

Fig. 3. Sheath cast in ground glass.

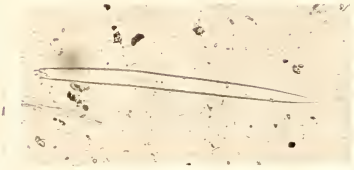

Fig. 4. Showing characteristics of sheath cast in sand.

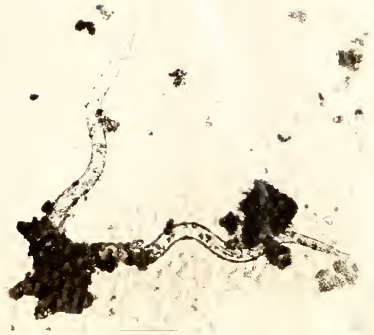

Fig. 5. Showing characteristics of sheath cast in a clay loam soil.

Fig. 6. Sheath cast by larva penetrating a mass of vegetable debris.

Fig. 7. Characteristics of larva killed in carbolic acid. Note omission of fluid at anterior end of esophagus.

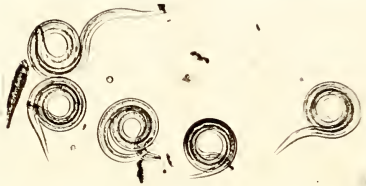

Fig. 8. Characteristics of larva killed in formalin.

Fig. 9. Characteristics of larva killed in corrosive sublimate.

Summary.

1. The second ecdysis of Necator americanus although a mechanical process is found to be distinctly unlike the first moult. The first ecdysis is due to the increasing size of the larva which bursts the skin, while after the larva reaches maturity it shrinks and the old skin is retained unless broken during the lateral flexures of the larva or by the mechanical aid from foreign substances.

2. The manner of unsheathing is largely dependent upon the character of the environment. In sand the anterior end of the sheath is worn down by friction against rough surfaces while in clay loams the sheath is retained by fine masses of soil, vegetation, etc., and becomes broken by the larva moving onwards.

3. The rate of unsheathing in a given number of larvae was found to be directly related to the texture of the soil.

4. Unsheathed larvae were found to be less resistant to unfavorable conditions than sheathed larvae.

5. In an infection experiment it was proven that unsheathed larvae are infective and become adults in the intestine within 6 weeks after the larvae penetrated the skin.

6. No difference was evident from data obtained from daily egg output calculations, in the infectivity of sheathed and unsheathed larvae.

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VI. FURTHER OBSERVATIONS ON THE MIGRATIONS AND THE POSITION OF INFECTIVE HOOKWORM LARVAE IN SOILS.

In a number of experiments made in Trinidad, British West Indies, during the summer of 1921 (Augustine, 1922) it was found that infective hookworm larvae do not actively migrate in the horizontal plane, and, as long as the soil is moist, remain on cr within its upper surface. The observations from these laboratory experiments were confirmed during the same summer by the field studies of Cort and Payne, (1922, a and b), who showed that the larvae are definitely localized at places of soil pollution.

During the following summer, 1922, further evidence on the failure of hookworm larvae to leave the place of development was obtained from a series of field experiments performed at Utuado, Porto Rico, on the length of life of the infective larvae (Augustine, 1923). In these experiments fresh stools containing abundant hookworm ova were placed in the centers of twelve wooden boxes, eighteen inches square and one foot deep, filled three-fourths full of moist soil. These boxes, some with vegetation, others without, were placed in different environments which offered a variety of conditions.

Examinations, with the unaided eye and with a hand

lens, of the surface of the soils ten days later revealed numerous aggregations of larvae protruding from the more prominent soil lumps and from small pebbles in all the boxes, where development was sufficiently great for such observations. Larvae were concentrated within the circles, about eight inches in diameter, where the stools had been placed, and in those boxes where development had been particularly heavy the centers appeared as if covered with hoar frost. A few larvae were found in the outer zones. The larvae were noted in these positions throughout the experiment in some of the boxes and in others as long as the numbers were sufficiently large for such observations.

One month after the larvae had reached maturity the soils of six of the boxes were examined. The central areas, about eight inches in diameter and one-half inch deep, were placed in separate units of the isolation apparatus from the soil of the remaining surface layers. In each case where larvae were present, the examinations showed that they were concentrated within the central zones. In one instance 11,460 mature hookworm larvae were isolated from this zone and only 2,020 from the remaining parts of the soil. From another box, 97,140 larvae were isolated from the middle area and 2,660 from without. No evidence of an active migration was indicated from any of these isolations. One month later, or two

months after the larvae had reached maturity, the soils of the remaining boxes were examined in a similar manner. While a great reduction had occurred in the numbers of larvae within this time, these examinations showed that their relative positions had not altered. From the surface layer of one box 520 mature hookworm larvae were isolated from the central portion and only 20 from the surrounding area. In another case, 60 larvae were isolated from the middle area and none were found outside of it. The position of the comparatively few larvae which were recovered from outside the polluted center need not be explained by any effort on the part of the larvae to leave their place of development. It is surprising that more larvae were not recovered from the outer zones since the boxes received no protection from the rains, except that offered by their environments. Certainly if infective hookworm larvae actually migrated actively from their place of development they would have been found evenly distributed all over the surface of these boxes.

These results corroborate my earlier experiments on the migration of infective hookworm larvae which were performed in Trinidad, British West Indies. They are also supported by the results of Cort, Payne, and Riley (1923)* ob-

* To be published.

tained from field studies in Porto Rico in four widely separated areas. These areas were entirely unlike and offered a wide variety of conditions; yet no where, was there observed any indication of an active migration of the larvae from localized centers of pollution. Where soil samples were taken but a few feet apart some would yield hundreds or even thousands of infective larvae while others would yield only a few or be entirely negative.

In order to determine the vertical distribution of the hookworm larvae in these boxes, all the soil was removed in the following zones and placed in units of the isolation apparatus, (1) upper half inch, (2) one inch, (3) one inch, (4) two inches, and (5) that amount which remained. The numbers of larvae isolated from each zone was determined by a dilution method which has been previously described (Augustine, 1923 b). Ths results of these isolations are summarized in Table I.

As will be seen from this table, the greatest number of hookworm larvae remain in the upper half inch of the soil and their numbers decrease rapidly with the increasing depth of the soil. In no instance were larvae isolated from the 4th zone, a depth between $2\frac{1}{2}$ and 4 inches.

Table 1. The Distribution of Infective Hookworm Larvae in Soil.

No.	Day stool was added to soil	Environment	Day examined	Larvae recovered from				
				1st ½"	2nd 1"	3rd 1"	4th 2"	remaining soil
4	July 13	Direct sun - with vegetation	Aug.23	32	0	0	0	0
5	" 10	Light shade - no vegetation	Aug. 18	13 ,480	2,150	414	0	0
7	" 14	Light shade - with vegetation	Aug. 26	10,820	1,400	20	0	0
8	" 14	"	Sept. 20	60	0	0	0	0
9	" 14	Dense shade - no vegetation	Aug. 22	2,300	60	42	0	0
10	" 14	"	Sept. 21	260	0	0	0	0
11	" 17	Dense shade - with vegetation	Aug. 26	99,700	4,6 00	60	0	0
12	" 17	"	Sept. 21	540	0	0	0	0

In all instances the soils were moist at the time of isolation except in box number 12 which appeared dry to a depth of 4 inches; yet larvae were recovered only from the upper zone. Green stalks and blades of grass from these boxes examined by means of the isolation apparatus were negative for hookworm larvae. These observations on the position of the infective hookworm larvae show that their natural position is on or close to the surface of the soil which makes easy their transfer to the human foot.

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VII. EXPERIMENTS ON THE FACTORS DETERMINING THE LENGTH OF LIFE OF INFECTIVE HOOKWORM LARVAE.

Introduction

For a successful hookworm campaign it is of primary importance to know whether or not abandoned pollution centers may remain, for a long period of time, as centers for reinfestation. In a series of laboratory experiments performed during the summer of 1921 in Trinidad, British West Indies, on the length of life of infective hookworm larvae in constantly moist soils, it was found that their life was limited to between six and seven weeks (Augustine, 1922). Similar findings were obtained by Cort and Payne during their field studies in Trinidad of the same summer (Cort and Payne, 1922). Further experiments were made the following summer (1922) at the Anemia Clinic, Utuado, Porto Rico, to determine some of the factors influencing the length of life of infective hookworm larvae. Both laboratory and field experiments were made, analyzing and duplicating, as far as possible, the factors or agencies existing in pollution centers, which were found around the houses and in the fields of this district. The importance of experiments to determine the length of life of hookworm

larvae under different temperature was early recognized. Such experiments were not feasible at the field laboratory and were carried out in Baltimore during the fall and winter of the same year.

Methods of Study

In all laboratory experiments in Porto Rico, the infective hookworm larvae were obtained from fresh stools mixed in previously heated soil and incubated six to seven days at outdoor temperature. For the experiments in Baltimore, the same method was employed in preparing the cultures, except that they were incubated for six days at 27° C. As Ancylostoma duodenale is almost unknown in Porto Rico, the larvae used were undoubtedly those of Necator americanus. The larvae used in the Baltimore series of experiments were also of Necator americanus.

Description of Culture House

Difficulties, due to improper housing, in early culture work made it necessary to erect a special room for this purpose (see Fig. I). As this building proved to be entirely satisfactory for such purposes, a short description of it may be of benefit to others having difficulties in obtaining pure cultures of hookworm larvae under tropical con-

Fig. 1. Culture house showing general construction.

ditions. Piles driven into the ground supported the floor one foot above the ground. The inside measurements were 6' x 6' x 6' and the only opening was a door at the front. The lower half of the walls were made solid with boards having overlapping edges and the upper half of the walls and ceiling were made of a closely woven muslin cloth tacked on the supporting studdings. The roof was of corrugated, galvanized tin sheeting. Two rows of shelves, one foot wide and one foot apart, accommodated the cultures. By raising the floor from the ground, the building was kept dry during the rainy season. It was rendered firm and substantial by making the lower half of the walls solid. The muslin is of distinct value over a solid board or screen wall in that it not only permits a free circulation of air, which eliminates any excess of odors arising from the cultures, but also excludes the smaller flies and insects which are not kept out by ordinary screening. Ants may be kept out by frequently washing the floor with creosote.

Technique of experiments

The methods of preparing the soils and counting the larvae in the experiments on the length of life of hookworm larvae to be described in this paper were the same as had been previously employed (Augustine, 1922, 23) and need

no further comment here. Two types of containers were used in the laboratory experiments, viz: (1) three ounce glass jars, the same as used in former experiments, and (2) tin pudding pans, six inches in diameter and two inches deep. For the field experiment, wooden boxes eighteen inches square and twelve inches deep were employed.

Length of Life of Infective Hookworm Larvae in Moist Soils

Table I gives the data from a series of experiments on the length of life of infective hookworm larvae in three distinctly different environments. The small glass jars were used in this experiment and the soil was kept moist. In the first experiment the containers were placed in a cleared spot in the center of a grass field and entirely exposed to direct sunlight throughout the day. The second, in light shade, was located on the east gallery of the laboratory. Here the units were protected from direct sunlight during the morning hours by the shade of nearby cocoanut palms, and from rains and the hot sun of the later hours of the day by the gallery roof. The third environment was in a wooded area at the edge of a banana grove. The vegetation here was particularly heavy and the leaves of the trees formed a veritable roof over head. This vegetation, with an abundant undergrowth of vines and low growing shrubs, afforded deep shade at all time.

Table I. Experiments showing reduction in numbers of infective hookworm larvae in constantly moist soils in different environments. All the experiments were started on June 24.

DENSE SHADE

Exp. No.	No. larvae placed in soil.	Day examined.	Larvae recovered Sheathed	Unsheathed.	Total	% reduction.
1	333	June 29	63	114	177	46.8
2	470	July 4	20	163	183	60.1
3	335	July 9	12	49	61	81.7
4	450	July 14	21	126	147	67.3
5	305	July 19	10	44	54	82.2
6	343	July 24	1	4	5	98.5
7	410	July 31	4	24	28	93.1
8	368	Aug. 7	8	8	16	95.6
9	467	Aug. 21	3	1	4	99.1
10	435	Aug. 29	0	0	0	100.

MODERATE SHADE

Exp. No.	No. larvae placed in soil.	Day examined.	Larvae recovered Sheathed	Unsheathed	Total	% reduction.
1	445	June 29	25	121	146	67.1
2	467	July 4	6	45	51	89.0
3	445	July 9	6	28	34	92.3
4	430	July 14	10	17	27	93.7
5	409	July 19	1	4	5	98.7
6	526	July 24	6	8	14	97.3
7	425	July 31	2	8	10	97.6
8	464	Aug. 7	0	1	1	99.8
9	435	Aug. 14	0	0	0	100.
10	446	Aug. 15	0	0	0	100.

DIRECT SUNLIGHT

Exp. No.	No. larvae placed in soil.	Day examined.	Larvae recovered Sheathed	Unsheathed	Total	% reduction.
1	645	June 29	0	1	1	99.8
2	358	July 4	0	0	0	100.
3	557	July 5	0	0	0	100.

The units of this experiment which were exposed to direct sunlight were returned to the gallery at night, and also in the day time during showers. The amount of evaporation in this environment was considerable and the soils required additional water many times each day. It was impossible to keep the soils in this environment constantly moist and at times the surface became slightly dry. The units placed on the gallery of the laboratory required no further protection than that supplied by the environment. The soils required a small amount of water each day. Banana leaves were placed over the units in the dense shade environment. These protected the soils from becoming covered with water during frequent showers and greatly reduced the amount of water evaporation.

The larvae used in the experiments were obtained from stools of a single individual and were isolated after six days of incubation. The soil was a sandy loam containing some humus and a little clay. Temperatures were read daily in each environment from maximum and minimum thermometers. These readings are summarized in Table II.

The results of these experiments (see Table I) clearly show that the length of life of infective hookworm larvae is largely determined by the character of the environment. Under dense shade a few larvae existed 7 to 9 weeks.

Table II. Summary of temperature readings for environments of Table 1. Readings are made from Fahrenheit thermometers.

Environment	Lowest	Highest	Average Minimum	Average Maximum.
Direct sun	60	120	64	116
Moderate shade	60	103	64	99
Deep shade	60	91	64	89

Under moderate shade an almost complete dying out was found after $5\frac{1}{2}$ weeks. A similar reduction occurred under direct sunlight within the first week. These data also show that in the environments most favorable to the existence of larvae the death rate is less rapid than in unfavorable environments. In dense shade, which is believed to be optimum, the rate of mortality is considerably lower than under light shade. A rapid dying out of the larvae occurred under direct sunlight; out of an original number of 645 larvae only one survived a week. While the environments were not entirely "natural," in that the soils were moist, it is evident that there was some factor, or a multiple of factors present in the direct sunlight and moderate shade environments which was lacking under deep shade. As moisture conditions were apparently optimum, except for the slight dying of the surface soil in the units exposed to direct sunlight, the higher rate of mortality in direct sunlight and moderate shade environments may be attributed chiefly to the higher and wider range of temperature existing within these environments. It is interesting to note in this connection that the dense shade situation of this experiment offered an environment almost exactly like that found in the coffee environment.

Length of Life of Infective Hookworm Larvae in Water Covered Soils.

Experiments, to determine how long infective hookworm larvae may live in water-covered soils, were made under the same environments as the previous experiments. The units of these experiments were prepared in the same manner as those of the preceding experiments except that from $\frac{3}{4}$ to 1 inch of water was constantly kept over the soil. The results of these experiments are summarized in Table III. These data, compared with the data of Table I, show a much greater mortality of larvae occurring in water-covered soil, in environments which were previously found to be favorable for their existence. Under dense shade, where infective hookworm larvae were found to exist for 7 to 9 weeks in moist soil, there was found to be a reduction of over 99% in ten days, and a complete dying out in less than 4 weeks in the soils covered with water. Approximately the same reduction was found among the larvae under the same conditions in light shade. All the larvae in direct sunlight died within the first week. No definite correlation can be made between the rapid dying out of the larvae and the varying character of the environments except, possibly, in the series exposed to direct sunlight, in which the more rapid death was, apparently due to high temperature. The

Table III. Experiments showing reduction of infective hookworm larvae in water-covered soil in different environments. Experiment started July 18. Approximately 318 larvae in each unit.

DENSE SHADE

Exp.No.	Day examined	Larvae recovered		Total	% reduction.
		Sheathed	Unsheathed		
1	July 28	2	0	2	99.3
2	Aug. 7	1	0	1	99.6
3	Aug. 22	0	0	0	100.
4	Aug. 23	0	0	0	100.

LIGHT SHADE

Exp.No.	Day examined	Larvae recovered		Total	% reduction.
		Sheathed	Unsheathed		
1	July 28	7	42	49	84.4
2	Aug. 7	2	4	6	98.1
3	Aug. 22	0	1	1	99.6
4	Aug. 29	0	0	0	100.
5	Aug. 30	0	0	0	100.

DIRECT SUNLIGHT

Exp.No.	Day examined	Larvae recovered		Total	% reduction.
		Sheathed	Unsheathed		
1	July 28	0	0	0	100.
2	July 29	0	0	0	100.

temperature of the water covering the soils of the units exposed to direct sunlight was from 2 to 10 higher than that of the surrounding air. This variation depended upon the intensity of the sunlight. This factor, however, cannot explain the high mortality occurring under the moderate and dense shade environments as no appreciable difference was noted between the temperature of the water and that of the surrounding air. It was noted, however, that where the mortality of the larvae was great the soil and water were teeming with protozoa, especially Infusoria. Larvae recovered, both from the superficial water and soil, were found to be partially or wholly disintegrated, with Colpidium feeding upon them.

Similar results were obtained from an experiment in which infective hookworm larvae were kept in tap water in open glass vials placed under the same three environments. Those larvae exposed to direct sunlight died within a week. The temperature of the water within these vials was always considerably higher than that of the surrounding air. All the larvae in moderate shade were dead and disintegrated within fifteen days. Some of the units, under deep shade, showed a reduction of 96 percent within 20 days while others gave a reduction of only 45 percent after one and a half months. No living larvae were found in any of the units

after two months. Here, as in the water covered soils, where larvae were found to have died quickly, Infusoria were present in great quantities. In several instances small flies had fallen into the vials and become covered with Saprolegnia. In other instances, larvae were completely surrounded by masses of fungi and in some cases the hyphae appeared to penetrate the sheaths of the larvae. The exact relation of the presence of protozoa, bacteria and fungi to the early death of infective hookworm larvae in water is not known. It is possible that the fungus surrounding the body of a larva so reduces the amount of oxygen for the larva that it perishes, or death may be due to some enzyme of the fungus which destroys the larvae.

It is of interest to note that similar observations have been recorded by Schüffner (1905, p. 685). Having observed that larvae of Ancylostoma in cultures were attacked by Infusoria which destroyed them within a few days, he came to the opinion that "as in the laboratory, so in nature, numerous enemies beset the young Ankylostomes and kill a large percentage." I have never seen Infusoria feed directly upon the bodies of living larvae. They feed upon the masses adhering to the larvae. It is only when the larvae are dead and partly disintegrated that they are food for these organisms.

Length of Life of Infective Hookworm Larvae in Alternating Moist and Drying Soils

Table I of paper number sixteen of this series, (Augustine, 1923) gives the data from an experiment on the length of life of infective hookworm larvae in alternating moist and drying soils. The technique and methods used in this experiment are discussed in the above mentioned paper and need not be repeated here. The results of this experiment showed a marked variation in the limits in the length of life of larvae developed from stools of different individuals. In three cases the larvae died within three weeks, in six cases within four weeks, in five cases within five weeks, in two cases within six weeks, in four cases within seven weeks and in two cases within eight weeks. In all instances 94 percent or more of the original number of larvae were found to have perished four weeks after the experiment was started.

The higher death rate of infective hookworm larvae in alternating moist and drying soils than in constantly moist soils (see Table I) can probably be explained by the differences in the activities of the larvae in these environments. In moist soils, larvae remain protruding singly or in clusters, from the soil particles, and unless stimulated are quite inactive save for the lateral flexure of their bodies. Repeated observations

made with a binocular microscope of the larvae on drying soils have shown that they leave their prominent positions and disappear within the upper layer of the soil. These observations were made upon larvae protruding from moist soils in syracuse watch glasses and were continued until the soils became noticeably dry. It was found in moist soils that the surface film of water completely covers the larvae, as it does the individual soil particles. As the water evaporates, this film becomes thinner and thinner and the pressure caused by it upon the larvae stimulates them and they move down between the soil particles. It was observed, however, that a large number of larvae fail to react to this stimulus until the film of water has become so reduced in thickness that migration apparently becomes impossible and they are then held fast to the particles of soil where they become victims of desiccation. Larvae, which were removed from the soil in this condition, ten minutes after all movement had ceased, and placed in water, failed to regain activity. When the soil was again moistened the survivors of the desiccation again occupied their former positions. The higher death rate and shorter length of life of infective hookworm larvae in alternating moist and drying soil may therefore be attributed both to the death of some of the larvae by drying and to the more rapid consumption of the stored

food due to the increased activity necessary for those that are able to follow the moisture as it recedes, and rises.

Length of Life of Infective Hookworm Larvae in Drying Soils

The question as to whether mature hookworm larvae can withstand desiccation is of greatest practical importance and has been a subject of much discussion. The literature on this point was thoroughly reviewed by Looss (1911). Through his own experiment, Looss proved that once the larvae were actually dry they were dead and could not be revived by the addition of water. These experiments were made in Egypt and consisted of allowing mature larvae in water to dry on a slide at an open window. We have, however, no experimental information on the length of life of infective hookworm larvae in soils which dry under natural conditions. The opinion has become current, that in all probability, some of the mature larvae at the close of the wet season of tropical countries may remain inactive in the soil throughout the dry months and be restored to their former activities with the coming of the rains of the following season. To determine how long mature hookworm larvae may live in drying soils under a variety of conditions and to estimate the minimum amount of water necessary in the soil to keep larvae alive, the following experiment was performed.

Moist soil was carefully weighed out in 54 gram lots and placed in sixty, three-ounce glass jars. To thirty of the jars infective hookworm larvae were added. The remaining thirty were used as controls to estimate the amount of water present in the soil at the time of the various examinations. The original amount of water in the soil was estimated to establish a standard with which to compare the amount of moisture present in the soils at the time of isolations. This was done by heating 54 grams of the moist soil for three-fourths of an hour at 60° C. The soil was rendered dust dry and weighed 43.2 grams. The difference in the weight of one of the controls and the weight of this dried soil was used as an estimate of the amount of water present in the units to be examined in which hookworm larvae had been added. Ten units with larvae and ten controls were placed in each of the environments which have been previously described. The units placed in direct sunlight were returned to the laboratory at night and during showers of the day. Those under moderate shade needed no further protection than that given by the nearby palm trees and the gallery roof. Large banana leaves were so arranged over the units placed under dense shade that they had complete protection from rains. These leaves also aided in reducing the amount of water evaporation.

Table IV gives the data obtained from these experiments on the length of life of infective hookworm larvae in drying soil. As will be seen from a study of this table, the rate of reduction of larvae increased with the decrease in the amount of moisture in the soil. Here again, it is shown that dense shade is most favorable for the existence of infective hookworm larvae. In this environment a few persisted in the drying soil for a little more than one month, while under moderate shade no larvae were recovered after ten days. In direct sunlight, an almost complete drying out of the larvae occurred within five days. No larvae were recovered from this environment after that time. As no larvae were isolated from soils which were naturally reduced to less than 3 percent of water as compared with the control in which the water was driven off by heating the soil, it may be assumed that this amount is the minimum of moisture which must remain in the soil to keep mature larvae alive. The rapid dying off of larvae under direct sunlight cannot be attributed wholly to the rapid evaporation of the water. It is probably due, in part, to the higher temperature of this environment, as shown in Table I. That the death of the larvae in most cases was due to desiccation rather than to the exhaustion of the stored food within the intestinal cells is

Table IV. Experiments showing reduction of infective hookworm larvae in drying soils under different environments. All experiments started June 29. Each unit contained 54 grams moist soil.

DENSE SHADE

No.	Original number larvae	Day examined.	Weight of soil in control	Percent of water in soil	Number larvae recovered	Percent reduction
1	366	July 4	50.9 g.	15.1	294 *	19.7
2	357	" 9	48.0 g.	10.0	187	47.6
3	390	" 14	45.8 g.	5.6	227	41.7
4	400	" 19	45.1 g.	4.1	62	84.5
5	372	" 24	45.1 g.	4.1	51	86.2
6	414	" 31	45.1 g.	4.1	15	96.3
7	401	Aug. 7	45.1 g.	4.1	0	100.
8	488	" 8	45.1 g.	4.1	0	100.

MODERATE SHADE

No.	Original number larvae	Day examined	Weight of soil in control	Percent of water in soil	Number larvae recovered	Percent reduction
1	354	July 4	46.9	7.8	202	42.9
2	342	July 9	44.8	3.5	118	65.4
3	337	July 14	44.6	3.1	0	100.
4	394	July 16	44.4	2.6	0	100.

DIRECT SUNLIGHT

No.	Original number larvae	Day examined	Weight of soil in control	Percent of water in soil	Number larvae recovered	Percent reduction
1	486	July 4	45.5	5.0	1	99.8
2	322	July 9	43.7	1.1	0	100.
3	398	July 10	43.6	0.9	0	100.

* The relation of the sheathed and unsheathed larvae in this experiment has been discussed in the text and in a previous paper (Augustine 1923 a).

evident, in that those last recovered were sheathed, well nourished forms, and had the appearance of newly developed larvae.

Influence of Temperature on the Life Span of Infective Hookworm Larvae.

The importance of more definite knowledge on the influence of temperature on the length of life of infective hookworm larvae in the soil was emphasized in my experiments performed in Porto Rico. These experiments showed both a lower death rate and a longer life for mature hookworm larvae than the Trinidad series of the previous summer (Augustine 1922). The only apparent difference was in the temperatures of the two countries. In Trinidad the daily temperature extremes were about 74° and 94° F., while in Porto Rico they were of 60° and 93° F. for the period in which the experiments were carried on. The maximum temperature, 93° F., was recorded only three times in Porto Rico during this time, and it usually varied between 90° and 92° (See Table VIII).

To determine the influence of temperature on the length of life of mature larvae in soil a series of experiments were made in which larvae in three ounce glass jars with moist soil, were exposed to the following temperatures, viz: 0° , 16° , 27° , room temperature varying from 20° to 31° , 35° and 40° C. The results of these experiments with the ex-

ception of the units exposed to 0° , 35° and 40° , are summarized in Table V. All the larvae exposed to 0° and 40° died within a week. Those exposed to 35° died within three weeks. The larvae used in these experiments were obtained from cultures incubated for six days at 27° . This table shows that a large number of larvae perished at each of the given temperatures within the first week of the experiment. A few of the larvae exposed to 16° persisted for fourteen weeks, while all the larvae at 27° died within eleven weeks. Over 99 percent of those kept at 27° perished at the end of nine weeks. A shorter length of life was found for the larvae kept at room temperature. This may be due to the changes in temperature during the night and day and, in part, to the occasional drying of the surface of the soils which was difficult to keep constantly moist. No difficulty was encountered in keeping the units in the constant temperature chambers moist as evaporation was reduced there to a minimum.

A second series of experiments was performed in which a single stool was divided into six 30 gram lots, which were cultured in soil. Three of the units were subjected to 27° , and the remaining three to 15° C. The soil of one unit of the series incubated at 27 degrees was placed in the isolation apparatus one week later. From this isolation 2672

Table V. Reduction of infective hookworm larvae when reared at 27°C. and kept at different temperatures.

345 larvae in each unit at 16°C.				335 larvae in each unit at room temperature - 20°-31°C.			341 larvae in each unit at 27°C.		
Unit No.	Time from beginning of experiment to examination of unit	No. larvae recovered	Percent. reduction	Time from beginning of experiment to examination of unit.	Number larvae recovered	Percent reduction	Time from beginning of experiment to examination of unit.	Number larvae recovered	Percent. reduction
1	1 week	42*	87.8	1 week	53	84.1	1 week	88	73.9
2	3 "	37	89.2	3 "	13	96.1	3 "	32	90.6
3	5 "	35	89.8	5 "	1	99.7	5 "	13	96.1
4	7 "	21	93.9	7 "	0	100.	7 "	12	96.4
5	9 "	17	95.0	7 "	0	100.	9 "	2	99.3
6	11 "	4	98.8				11 "	0	100
7	14 "	6	98.2				11 "	0	100
8	17½ "	0	100.						

* Both sheathed and unsheathed larvae were found but are not recorded in the table because there was nothing significant in the proportions.

mature hookworm larvae were isolated. Two weeks were allowed before isolating the larvae from one of the units reared at 16° . (Looss, 1911, p. 387, has found that larvae need 13 days to attain maturity at temperatures between 15° and 17°). This isolation yielded 2510 mature larvae. The number of larvae obtained from these isolations were used as an estimate of the numbers in the remaining units; and the percentages of reductions computed from them. The results obtained from this experiment are given in Table VI. Judging from these data, (See Tables I and VIII) the longer life of the infective hookworm larvae, as found in Porto Rico, may be attributed to the cooler temperatures of the district in which the experiments were performed.

As will be seen from this table (Table VI) the reduction of the numbers of larvae reared and maintained at 16° is considerably less than those reared at higher and later subjected to lower temperatures (Table V). This may in part be due to their increased activity in passing through the isolation apparatus and in part to the change in temperature. The larvae kept at 16° C. had a noticeably greater supply of stored food material in their intestinal cells than those which had been kept at 27° C. Oliver (1910) observed that larvae developing at lower temperature were

Table V. Comparison in the percentages of reduction of hookworm larvae at 16° and 27° C. Experiment started January 29, 1923.

No.	Temperature	No. larvae per unit	Day of 1st examination	No larvae isolated	% reduction	Day of 2nd examination	No. larvae isolated	% reduction
1	16°	2510	3-13-23	2080	16.1	4-4-23	2040	18.2
2	27°	2672	3-13-23	340	87.2	4-4-23	2	99.8

much stronger and more vigorous than larvae hatched from eggs incubated at higher temperatures. I have found no apparent differences in the character of larvae which have developed at different temperatures, yet my experiments on the length of life of infective larvae tend to support his view.

Field Experiments on the Length of Life of Hookworm Larvae under Different Environmental Conditions

The following experiment was performed to determine the length of life of hookworm larvae under natural conditions of development in different environments. Three distinct areas were selected. The first was located along an open strip at the edge of a forage grass field and was exposed to the sun throughout the day (see fig. 2). The second area was within a cluster of cocoanut palm and orange trees. Direct sunlight shown here for a short time only in the late afternoon (see fig. 3). The third area was located in a thicket which afforded deep shade (see fig. 4). A description of this environment has been given in an earlier paragraph of this paper.

Fresh stools, or stools less than twelve hours old, obtained from a single individual were used exclusively in this experiment. The character of the specimens varied but slightly. They were, for the most part, quite mushy. They

Fig. 2. Field experiments in direct sunlight. Control cultures are to the right of each box.

Fig. 3. Field experiments in light shade. Control cultures are at the right of the boxes.

Fig. 4. Field experiments in deep shade. Control cultures to the left of the boxes.

contained a considerable amount of undigested vegetable matter such as beans and fruit shreds and were slightly acid. Each stool was weighed when received at the laboratory. Four fifths of a specimen was placed in the center of a wooden box, eighteen inches square and twelve inches deep, filled three-fourths full of moist sandy loam soil. The specimen was then worked into the soil. The remaining portion of the stool was cultured separately in a similar manner, but in perforated tin basins, and placed beside its corresponding box (See figs. 2, 3 and 4). The number of larvae isolated from this culture, eight days later, multiplied by 4 was used as an estimate of the number of larvae having developed in the box. In all twelve boxes were prepared, six with no vegetation and six which had been previously sodded with forage grass. Two boxes of each were placed in the three environments together with their corresponding controls. Boxes, numbered 2, 4, 6, 8, 10, and 12 and their controls were kept moist until the larvae were isolated from their controls. Numbers 1, 3, 5, 7, 9, and 11 and their controls were left entirely to the factors existing in their respective environments. Frequent examinations of the boxes showed the soils of those units placed in deep shade were always moist. The surfaces of the soils of those in light shade were oc-

casionally dry but never during the time of development of the larvae. The units exposed to direct sunlight were frequently dry aften to a depth of three inches.

The weather reports for the Utuado district, from July 12 to September 21, 1922, the time the field experiments were made, are given in Table VII. I am indebted to the officers of the local Weather Bureau at Utuado, Porto Rico for these data.

The data obtained from these experiments (see Table VIII) show that soils without vegetation are less favorable for the development and existence of infective hookworm larvae than soils with vegetation. In soils with no vegetation the rate of reduction was found to be greater and the length of life of the larvae shorter than in soils with vegetation. In box 5, for example, with no vegetation (see Table VIII) a reduction of 83.6 percent from the original number of larvae occurred one month after the larvae had reached maturity, while in box 7, within the same environment, light shade and with vegetation a reduction of only 60.4 percent occurred within a similar period of time. A similar comparison of the reduction of larvae in soils with and without vegetation is found with boxes 9 and 11 under dense shade. This same relation was found to hold true after a period of two months. Box 6, with no vegetation was nega-

Table VII. Weather Reports for Utuado, Porto Rico, Observations made by Martinez and Co. Utuado, Porto Rico.

Day	Temperature			Amount Precipitation.
	Maximum	Minimum	Range	
July 12	90	63	27	1.20
13	89	63	26	
14	86	64	22	
15	93	64	29	
16	92	65	27	
17	88	66	22	.50
18	90	64	26	
19	87	63	24	
20	88	64	24	
21	95	63	32	
22	88	65	23	.95
23	89	66	23	
24	90	66	24	
25	90	67	23	
26	90	66	24	
27	88	66	22	
28	91	65	26	
29	89	65	24	
30	89	65	24	
31	91	65	26	
August 1	90	64	26	
2	89	64	25	
3	90	65	25	
4	90	65	25	
5	90	66	24	2.00
6	89	67	22	
7	91	65	26	
8	90	66	24	
9	91	65	26	
10	93	65	27	.90
11	87	66	21	
12	85	65	20	
13	90	65	25	
14	90	65	25	.60
15	88	66	22	
16	88	66	22	
17	90	66	24	
18	92	66	26	
19	88	66	22	
20	93	66	27	

Table VII continued.

Day	Temperature			Amount Precipitation
	Maximum	Minimum	Range	
August 21	90	60	30	1.00
22	92	60	32	
23	90	61	29	
24	88	61	27	
25	88	61	27	
26	89	61	28	
27	89	61	28	
28	90	61	29	
29	90	63	27	.80
30	89	63	26	
31	90	63	27	1.20
September 1	90	64	26	.80
2	90	64	26	
3	88	65	23	.75
4	90	65	25	
5	90	65	25	
6	90	65	25	
7	87	65	12	
8	92	65	27	
9	88	65	23	.80
10	87	66	21	.85
11	90	66	24	
12	90	66	24	
13	80	66	14	
14	89	66	23	
15	89	65	24	.40
16	97	65	32	.50
17	90	65	25	.55
18	92	65	27	
19	91	65	26	1.25
20	90	65	25	
21	91	65	26	2.00

tive for hookworm larvae, while 60 larvae or .2 percent of the original number were isolated from box 8 which had vegetation. Again, box 10, without vegetation, under dense shade, was negative after a period of 2 months, while box 12 with vegetation and within the same environment, still harbored 540 or .6 percent of the original number of infective larvae.

The reduction of mature hookworm larvae, as shown from the data of Table VIII, was followed with the unaided eye and with a hand lens. In those boxes where development had been particularly heavy the larvae appeared in concentrated masses within the central portion of the surface of the soils where the stools had been placed and appeared as hoar frost on the pebbles and prominent soil particles 8 days after the experiment was started. One month after the larvae had reached maturity a marked reduction was noted in their numbers, which continued to increase with time until no larvae could be found in this manner. In box 12, however, with vegetation, and in dense shade, a few single larvae were found on the surface of the soil up to the day of final examination.

It is evident from these data, (Table VIII) that a few larvae may reach maturity in direct sunlight but soon perish. Time was not available to continue these field ex-

Table VIII. Comparison of the development and length of life of hookworm larvae in soil.

Box No.	Day stool was added to soil	Environment	No. larvae isolated from control after 8 days	Approx. no. larvae in box	Day of examination of soil in boxes	No. larvae recovered from soil in boxes	Percent. reduction.
1	July 12	Direct sun- no vegetation	0	150*	Aug. 22	0	100
2	" 12	"	981	3,924	Sept. 5	0	100
3	" 13	Direct sun - with vegetation	1,052	4,208	Aug. 23	32	99.0
4	" 13	"	2,040	8,160	Sept.14	0	100
5	" 10	Light shade - no vegetation	24,540	98,160	Aug. 13	16,044	83.6
6	" 10	"	26,400	105,600	Sept.20	0	100
7	" 14	Light shade - with vegetation	7,740	30,960	Aug. 26	12,240	60.4
8	" 14	"	9,148	36,592	Sept. 20	60	99.8
9	" 15	Dense shade - no vegetation	2,420	9,680	Aug. 22	2,402	75.1
10	" 15	"	12,042	48,168	Sept.21	0	100
11	" 17	Dense shade - with vegetation	47,530	190,120	Aug. 26	104,360	45.1
12	" 17	"	24,920	99,680	Sept. 21	540	99.4

* 150 unsheathed, poorly nourished larvae were isolated from soil taken from the upper center of this box. These larvae were returned to same box.

periments long enough to determine the time of complete reduction dying out of the larvae in the moderate and dense shade environments. However, the larvae from the last isolations were attenuated, inactive forms and, judging from previous observations, would have died within a short period of time. That a reduction of over 99 percent may occur two months after the larvae have reached maturity is significant and corroborates the results of my earlier Porto Rican experiments which were started with mature larvae (see Table I).

Discussion

While many of the previous investigators have kept infective hookworm larvae alive for months and years in artificial media under laboratory conditions, nothing was known concerning the limits of their life in soil under natural conditions until an apparatus was devised, with which the larvae could be isolated from soil of considerable quantity (Baermann, 1917). By the use of this apparatus, with slight modifications (Cort, Ackert et al, 1922), it has been possible to study the larvae in their natural environment and obtain definite information on their life span under varying conditions. Under favorable conditions the length of life of infective hookworm larvae apparently

depends upon the rate of consumption of the stored food within the intestinal cells. This being the case, we may assume that the exhaustion of this supply sets a limit to the life of the individual. The length of life then depends on whether the supply of food is consumed slowly or rapidly and any factor or multiple of factors which would tend to increase the activity of the larvae would tend to shorten their lives. That this factor actually operates is suggested by the more rapid death of the larvae at higher temperatures and those subjected to alternate wetting and drying.

While we have no experimental evidences it seems probable that the life span of mature larvae is shortened by their being washed about during the frequent showers, which are common in the tropics. It is possible that they become washed out of localities which are favorable for their existence into less favorable environments where their life will be limited to a few days. They may also be carried to positions where they cannot be picked up by man. That mature hookworm larvae cast their protective sheaths in the soil is significant in that they are immediately rendered more susceptible to adverse conditions, (Augustine, 1923 a) and their life shortened. It is generally supposed that mature

hookworm larvae have but few or no natural enemies. My observations, on larvae in open water and in water covered soils in which fungi, bacteria and protozoa were present in abundance, suggest that such enemies may exist under natural conditions.

Probably one of the most important factors in reducing soil infestation is the washing of the heavy rains. The operation of this factor under field conditions is well illustrated by the situation of Area C of the Porto Rico field studies (Cort, Payne, and Riley *). This point is also illustrated by the shorter life of the larvae which were washed into the drains of the cane area studied in Trinidad (Cort and Payne, 1922). The rapid drying of the soil under field conditions is also an important factor in the reduction of soil infestation. The importance of this factor in Porto Rico is shown by the rapid reduction of soil infestation in Area A of the Porto Rican field studies (Cort, Riley, Payne, and Hill).* In fact, in all the studies in which soil surveys for infective hookworm larvae have been made, environments which were exposed to rapid drying have shown but slight soil infestation, even where there was a heavy soil pollution from infected individuals.

* Unpublished.

This evidence on the rapid reduction of soil infestation and the limited length of life of infective larvae in their natural environments, confirmed both by laboratory and field experiments, is significant in hookworm control work in its relation to the possibilities of reinfestation. This relationship has been emphasized by Cort (1922) in a recent analysis of the factors in hookworm control. It would seem essential that the factor of soil infestation should receive more consideration in control work and that the control measures of treatment and sanitation should be so related to the length of life of hookworm larvae in the soil that the danger of reinfestation would be reduced to a minimum.

While these data show a rapid dying out of hookworm larvae under tropical environments, it is well to call attention again to the different results of former workers who have reported a much longer span of life for mature larvae when kept in water, fecal cultures, etc. in temperate regions, and to the variations found under different conditions in my own experiments. It is therefore necessary to increase the numbers of observations on the length of life of hookworm larvae under a variety of conditions in different parts of the world, especially in temperate and sub-tropical climates before it will be possible to draw any far-reaching generalizations. The evidence brought

out in the Trinidad and Porto Rico work, both from laboratory experiments and field studies makes it seem extremely probable that under tropical conditions in general, the length of hookworm larvae in the soil is comparatively short and that treatment or sanitation in any group of people rapidly leads to a natural sterilization of the soil.

Summary

1. Laboratory and field experiments were performed testing the factors which influence the length of life of infective hookworm larvae.
2. In moist soils in Porto Rico, a few infective hookworm larvae lived for 8 weeks in deep shade and 6 weeks in light shade. Under direct sunlight an almost complete dying out occurred within 5 days.
3. Infective hookworm larvae were found to die rapidly in water covered soils. The early death of the larvae is associated with the presence of fungi, bacteria, and protozoa.
4. In drying soils in dense shade a few mature larvae were recovered one month after the experiment was started. In light shade, where evaporation of water was rapid, all were dead after about 12 days.

5. The alternating drying and moistening of soils hastens the death of hookworm larvae.

6. Temperature has an important bearing on the length of life of mature hookworm larvae. At 27° C. over 99 percent of the original number of larvae perished after 9 weeks; at 35° C. all were dead within 3 weeks. When larvae are exposed to cooler temperatures, 15° C., the rate of reduction is considerably less than at 27° C., and their length of life is longer. 0° C. was found to be unfavorable for the existence of infective hookworm larvae and they perished within one week.

7. Soils with vegetation are more favorable for the development and life of infective larvae than soils without vegetation.

8. Environments exposed to direct sun of the tropics are unfavorable for hookworm development and the few larvae which reach maturity die within a few days.

9. Dense shade affords an optimum environment for the development and continued existence of mature hookworm larvae. Approximately .6 percent of the original number of infective larvae were recovered 9 weeks after the deposition of a stool, from an infected individual, upon the soil.

10. Washing of the soil by heavy rains and the drying of the soil, under field conditions, are important factors in reducing soil infestation.

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Biography

Donald Leslie Augustine - Born in Kasson, Minnesota, January 1, 1895.

Education - Diploma, Kasson High School, 1913; S. B., Macalester College, 1918; Summer Session of Biological Station, University of Michigan, 1916; Special Student, Zoology, University of California, 1918; U. S. Army, Laboratory Car "Metchnikoff" and Department Laboratory, Southern Department, 1918-1919; Graduate Student, Zoology, University of Minnestoa, 1919-1920; Johns Hopkins University, School of Hygiene and Public Health, 1920. Member of the Commission to investigate control measures for hookworm disease, Trinidad, 1921, Porto Rico, 1922.

Teaching Experience - Assistant in Biology, Macalester College, 1916-1918; Teaching Fellow in Zoology, University of California, 1918; Adjunct Professor of Biology, Macalester College, 1919-1920; Assistant in Helminthology, Johns Hopkins University, 1920-1923.

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